**The Dissertation Committee for Michael Don Dennis Certifies that this is the approved version of the following dissertation:**

# Phosphorylation of Plant Translation Initiation Factors by CK2

**Committee:**

Karen S. Browning, Supervisor

Dean R. Appling

David E. Graham

Arlen W. Johnson

Christian P. Whitman

# Phosphorylation of Plant Translation Initiation Factors by CK2

**by**

**Michael Don Dennis, B.S.**

**Dissertation**

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

**Doctor of Philosophy**

**The University of Texas at Austin**

**May 2008**

## Dedication

This dissertation is dedicated to my greatest blessing, my wife, who shared the many risks and sacrifices required for its completion. Amanda's unconditional love and encouragement throughout this process has made the difficult journey possible. For her unending support, and for being everything that I am not, she has my eternal love.

## Acknowledgements

I would like to express thanks to the numerous people who have made my journey as a scientist possible. Throughout my education, I have had the good fortune to cross paths with many brilliant minds that have inspired me to think critically about the world around me. While an undergraduate biology major at Centre College, I was fortunate to be guided by Dr. Christine Barton, who always encouraged me to remain flexible in my research and pursue my own ideas. I have an extreme gratitude for my dissertation advisor, Dr. Karen Browning, who took me into her lab 5 years ago. Under her guidance, I have taken my first steps in what is hopefully a long career as a successful biochemist. I would also like to thank all my colleagues in the Browning lab who have assisted in my professional development in far too many ways to recount. I would like to specifically thank Laura Mayberry, Dr. Andrew Lellis, and Dr. Leah Allen, who were always willing to share their expertise and provide practical advise. I am particularly indebted to Dr. Maria Person, who contributed numerous days to the preparation of samples and the analysis of phosphopeptide mass spectra. Prior to her collaboration, attempts at proteomic analysis were terribly unsuccessful, and her expertise made much of this dissertation possible.

I am eternally grateful for my family who instilled in me the courage and curiosity to always question what is possible and to never give up pursuit of my goals. None of this would have been possible without their love and support. My wife, whom this

dissertation is dedicated to, has been a constant source of patience, encouragement, and strength throughout this journey.

# Phosphorylation of Plant Translation Initiation Factors by CK2

Publication No._____

Michael Don Dennis, Ph.D.

The University of Texas at Austin, 2008

Supervisor: Karen S. Browning

Protein kinase CK2 phosphorylates wheat eIF2, eIF3, eIF4B, eIF5 and three 60S ribosomal proteins. The substrate specificity of CK2α toward various plant initiation factor substrates was altered *in vitro* through holoenzyme formation in the presence of regulatory β-subunits. This presents a potential mechanism through which the differential expression and sub-cellular distribution of CK2 β-subunits could regulate phosphorylation of various CK2 substrates in plants. Our analysis of initiation factor phosphopeptides produced by *in vitro* phosphorylation identified 20 CK2 phosphorylation sites in eIF2α, eIF2β, eIF3c, eIF4B, and eIF5. Native wheat eIF5 was prepared in the presence of phosphatase inhibitors and analyzed by mass spectrometry. Native wheat eIF5 was determined to be a phosphoprotein containing at least 3 phosphorylation sites. The C-terminal CK2 site (S451) of native eIF5 was completely phosphorylated, and tryptic fragments containing the other *in vitro* CK2 two sites (S209, T240) also appear to be partially phosphorylated.

Many of the CK2 phosphorylation sites identified are in conserved binding domains of the yeast multifactor complex (eIF1/eIF3/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$). This observation lead to the hypothesis that CK2 phosphorylation may regulate the formation of plant multifactor complexes. The results presented here suggest that plant initiation factors are capable of forming complexes similar to those previously reported in yeast. The *in vitro* interaction of initiation factors within these complexes appears to be enhanced by phosphorylation of eIF2, eIF3c, and eIF5 by CK2. Site-directed mutagenesis of eIF5 to remove CK2 phosphorylation sites not only prevents the CK2 mediated increase in interaction with eIF1, but also resulted in reduced stimulation of translation initiation *in vitro.*

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1: Introduction

## 1.1 PROTEIN BIOSYNTHESIS

Protein biosynthesis is a critical step in gene expression through which the genetic information contained within the base sequences of a messenger RNA (mRNA) is translated into a polypeptide of amino acids. The molecular machine responsible for this process of translation is known as the ribosome. Translation can be broken down into three steps: initiation, elongation, and termination. During translation initiation, a variety of initiation factors participate in the assembly of a ribosome onto an mRNA. During polypeptide elongation, the ribosome moves along the mRNA, adding sequence specified amino acids to the growing polypeptide chain via a transpeptidation reaction that is catalyzed by the 23S rRNA of the large ribosomal subunit. Termination is then triggered when the ribosome reaches the "stop" codon, which binds release factors. This process leads to the release of the polypeptide and the dissociation of the ribosome into its two subunits.

### 1.1.1. Translation Initiation

Translation initiation is typically the rate-limiting step in eukaryotic polypeptide biosynthesis, culminating in the assembly of a functional 80S ribosome with the initiation codon of an mRNA base-paired to the Met-tRNA$_i^{Met}$ anticodon in the ribosomal peptidyl (P) site (recently reviewed in (Pestova et al., 2007). This process requires at least 11 eukaryotic initiation factors (eIFs), mRNA, tRNAs, and energy in the form of ATP and GTP hydrolysis. Translation begins with the assembly of the 43S preinitiation complex

(PIC). For this to take place, the eIF2-GDP complex that results from each round of translation initiation must be recycled to eIF2-GTP in a reaction that is catalyzed by the guanine nucleotide exchange factor (GEF) eIF2B (Nika et al., 2000). This exchange leads to the binding of Met-tRNA$_i^{Met}$ and the formation of a ternary complex (TC) consisting of eIF2-GTP-Met-tRNA$_i^{Met}$ (Erickson and Hannig, 1996). The eIF2 ternary complex, along with eIF1, eIF1A, eIF3, and eIF5, is then assembled onto the small 40S ribosomal subunit to form the 43S pre-initiation complex (Hinnebusch et al., 2007). Biochemical data from yeast suggests that a multifactor complex (MFC) consisting of eIF3/eIF1/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$ can exist free of the ribosome, allowing these factors to be simultaneously loaded onto the 40S ribosomal subunit (Asano et al., 2000). The assembly of this complex and its impact on translation will be explored further in later sections of this manuscript.

The 43S pre-initiation complex is loaded onto mRNA by eIF4F, eIF3, and poly(A) binding protein (PABP), leading to the formation of the 48S pre-initiation complex (Pestova et al., 1998). Unlike the prokaryotic 30S ribosomal subunit, the eukaryotic 40S subunit does not directly locate to the position of the AUG codon of mRNA. The recruitment of the 40S ribosome onto eukaryotic mRNAs generally occurs via the 5′-$^{7m}$GpppN "capped" end of the mRNA and requires the initiation complex to scan for the proper initiation site context (Pestova et al., 2001). The initiation factor eIF4F consists of the 5′ cap-binding protein eIF4E bound to the N-terminal region of the scaffolding protein eIF4G. Poly(A) binding protein is simultaneously bound by both eIF4G and the poly(A) tail of the mRNA to enhance cap binding by eIF4E (Borman et al., 2000). The C-terminal region of eIF4G recruits the RNA helicase, eIF4A, which is

responsible for the ATP-dependent unwinding of the secondary structures in the 5′-untranslated region (UTR) of the mRNA. The eIF4G subunit is also responsible for binding the RNA-binding protein eIF4B, which contains several RNA binding motifs and helps to stabilize binding of eIF4A and promote the ATP-hydrolysis that is required in scanning (Cheng and Gallie, 2006).

Once loaded onto the mRNA, the pre-initiation complex will scan 5′ to 3′ in search of an AUG initiation codon, and base pair with the anticodon of the Met-tRNA$_i^{Met}$ (Kozak, 1978). eIF1 and eIF1A play an important role in this process (Pestova et al., 1998). Once base pairing has taken place, GTP hydrolysis by eIF2 is triggered by the GTPase-activating protein (GAP), eIF5 (Das et al., 2001). This event causes eIF2-GDP to release the Met-tRNA$_i$. The ribosome dependent GTPase eIF5B then stimulates the release of eIF2-GDP, which dissociates from the ribosome complex along with eIFs 1, 1A, 3, and 5 (Pestova et al., 2007). Once these factors have dissociated, eIF5B facilitates the binding of the large 60S ribosomal subunit, which leads to eIF5B stimulated GTP hydrolysis and its dissociation from the ribosome (Pestova et al., 2000). The result of this process is a functional 80S ribosome assembled onto the mRNA with the Met-tRNA$_i$ base-paired to the initiation codon in the ribosomal P-site.

### 1.1.2. The Multifactor Complex

It has been shown that a stable multifactor complex (MFC) consisting of eIF3/eIF1/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$ exists independently of ribosomal subunits and serves as a critical translation initiation intermediate *in vivo* (Asano et al., 2000; Asano et al., 2001a; Valásek et al., 2003). The interaction of factors within the multifactor

complex appears to play an important role in optimal 43S pre-initiation complex assembly in yeast (Hinnebusch et al., 2007). Mutations that disrupt the protein-protein interaction regions in the eIF3c-N-terminal domain, eIF5-C-terminal domain, and eIF1 reduce the association of eIF2 with 40S subunits and reduce the translation of GCN4 mRNA (Asano et al., 2000). GCN4 is a transcriptional activator of amino acid biosynthetic genes, which allows cells in nutrient-poor environments to increase the production of amino acids while reducing rates of general protein synthesis. The effect of mutations that disrupt critical binding regions is negated by the overexpression of the eIF2 ternary complex (Asano et al., 2001a). Proper multifactor complex formation not only plays a critical role in recruitment of the ternary complex to 40S ribosomes, but is also suspected to mediate the proper selection of AUG start codons (Valasek et al., 2004).

A growing body of evidence suggests that eIF3 may function as the central hub in the coordination of translation initiation (Hinnebusch, 2006). Approximately 700 kDa in size, eIF3 is the largest and most complex initiation factor. Yeast eIF3 consists of 8 subunits, 5 of which assemble the "core" (TIF32/eIF3a, PRT1/eIF3b, NIP1/eIF3c, TIF35/eIF3g, TIF34/eIF3i) that is essential for translation in yeast (Phan et al., 1998). Budding yeast eIF3 contains substoichiometric amounts of the nonessential HCR1/eIF3j, which regulates the function of the essential core by controlling access of the mRNA-binding cleft (Hinnebusch, 2006; Fraser et al., 2007). While evidence exists that "non-core" subunits are not essential for global translation initiation, they do appear to play an important role in proper cellular function (Bandyopadhyay et al., 2002). Plant and mammalian eIF3 contain 12 and 13 subunits, respectively (Hinnebusch, 2006).

Interestingly, deletion analysis of mammalian eIF3 subunits recently revealed an unexpected essential core consisting of 6 subunits (eIF3a, eIF3b, eIF3c, eIF3e, eIF3f, eIF3h; (Masutani et al., 2007).

In accordance with its structural complexity, eIF3 plays an important role in ensuring proper translation initiation at most steps in the initiation pathway. eIF3 prevents premature binding of the ribosomal subunits, enhances eIF2-GTP-Met-tRNA$_i$ ternary complex formation, directs the interactions of multiple eIFs during their assembly into pre-initiation complexes, improves mRNA recruitment to the 43S pre-initiation complex, and participates in both scanning and AUG recognition (Hinnebusch, 2006). In addition to the ribosomal 40S subunit, studies have shown that eIF3 interacts with eIFs 1, 1A, 2, 4B, 4G, and 5 (Hinnebusch et al., 2007). Interestingly, eIF3 subunits have also been found to associate with the COP9 signalsome and 26S proteasome, suggesting a potential link to other cellular processes (Karniol et al., 1998; Yahalom et al., 2001).

eIF2 is a heterotrimer of approximately 143 kDa consisting of α, β, and γ subunits. Genetic studies in yeast have demonstrated that all 3 subunits are required for cell viability (Donahue et al., 1988; Cigan et al., 1989; Hannig et al., 1993). The eIF2γ subunit not only serves as an intermediary binding to both the α and β subunits, but also functions in guanine nucleotide and Met-tRNA$_i^{Met}$ binding (Erickson and Hannig, 1996). The eIF2β and eIF2α subunits do not appear to directly interact with one another (Schmitt et al., 2002). eIF2β contains multiple tracts of lysine residues (K-boxes) in the N-terminal portion of the protein that likely functions in binding mRNA and other initiation factors (Asano et al., 1999). In addition the eIF2β C-terminus possesses a zinc finger motif that is required for initiator codon selection (Donahue et al., 1988). In mammalian

cells, the eIF2α subunit functions in interaction with the guanine exchange factor (eIF2B) playing a critical role in the regulation of eIF2 activity. Phosphorylation of S51 by eIF2α kinases converts eIF2α from a substrate to a competitive inhibitor of eIF2B, and thus functions as a key mechanism for the repression of global protein synthesis.

eIF5 is a monomeric protein of approximately 50 kDa which plays an essential role in translation initiation as a GTPase activating protein (GAP; (Das et al., 2001). The N-terminus of eIF5 contains the GAP domain, which interacts with the eIF2γ subunit to stimulate the hydrolysis of GTP (Alone and Dever, 2006). The C-terminal region of eIF5 contains an 8-alpha helical HEAT domain, which is responsible for binding eIF1, eIF2β, and eIF3c in the yeast multifactor complex and eIF4G in the 48S pre-initiation complex (Das and Maitra, 2000; Das et al., 2001; Yamamoto et al., 2005). Binding of eIF4G is believed to displace binding of eIF1 and eIF2, yet the eIF5-C-terminal domain can simultaneously bind eIF4G and eIF3 (Yamamoto et al., 2005). In mammals, the acidic C-terminus may also function in binding eIF5B (Marintchev and Wagner, 2004); however, it is not clear if this is the case in yeast and plants. It also appears that eIF5 may play a role in AUG selection through an interaction with eIF1A, which shifts the 43S complex from a scanning competent state to a scanning incompetent state (Maag et al., 2005).

eIF1 is a small 12 kDa protein that plays an important role in ensuring the fidelity of translation via the destabilization of ribosomal complexes assembled at incorrect start codons (Pestova and Kolupaeva, 2002). eIF1 regulates both the GAP activity of eIF5 and the release of the $P_i$ generated by eIF2-GTP hydrolysis, which triggers factor release from the 48S pre-initiation complex (Valasek et al., 2004). Mammalian and yeast eIF1 exhibit

a globular α/β core and an unstructured N-terminal tail (Fletcher et al., 1999; Reibarkh et al., 2008). Interestingly, this is structurally similar to the N-terminal domain of eIF5, and thus a model has been suggested where eIF1 antagonizes the interaction of eIF5 with eIF2 in the absence of proper AUG selection (Conte et al., 2006).

Using a variety of *in vitro* binding assays, two-hybrid analysis, and *in vivo* purification of affinity tagged subunits an extensive analysis of the subunit interactions within the multifactor complex (MFC) has resulted in a provisional model of the yeast MFC (summarized in (Hinnebusch et al., 2007). An interaction map based on data from these studies can be seen in Figure 1.1. These studies demonstrate that the 3 largest subunits of eIF3 (TIF32/eIF3a, PRT1/eIF3b, NIP1/eIF3c) each contain a binding site for the other two subunits. The extreme C-terminus of eIF3b was additionally found to interact with the other two core subunits TIF34/eIF3i and TIF35/eIF3g. The regulatory HCR1/eIF3j subunit in turn binds both the C-terminus of eIF3a and the N-terminus of eIF3b. The NIP1/eIF3c N-terminal domain plays a particularly important role in binding to other initiation factors in the context of the multifactor complex. The NIP1-NTD was found to bind directly to both eIF1 and eIF5, and it is suspected that this binding coordinates the interaction between eIF1 and eIF5 that is responsible for the inhibition of GTP hydrolysis at non-AUG codons (Valasek et al., 2004).

In addition to eIF3c, the CTD of eIF5 serves as a core subunit in the assembly of the multifactor complex. The CTD of eIF5 simultaneously binds to both the N-terminus of eIF3c, the N-terminal K-boxes of eIF2β, and eIF1 (Yamamoto et al., 2005). This creates an indirect link between eIF3c and eIF2β, which is further supported by the direct interaction of eIF3a with eIF2β and the multiple interactions of eIF1 (Valasek et al.,

2002). In yeast, eIF1 interacts directly with the NTDs of both eIF2β and eIF3c, in addition to binding to the CTD of eIF5 (Valasek et al., 2004; Yamamoto et al., 2005; Reibarkh et al., 2008). It is not clear however, if all of these interactions are conserved in other eukaryotes, as NMR was unable to detect any interaction between human eIF1 and a recombinant form of human eIF5 (Fletcher et al., 1999).

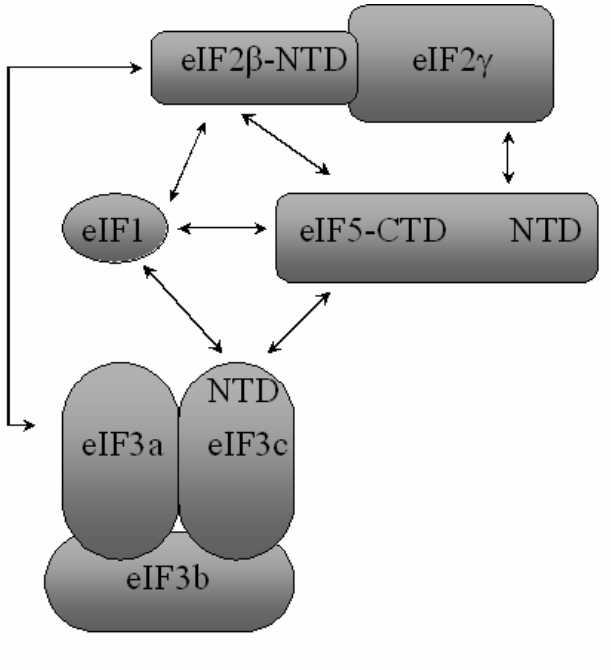



**Figure 1.1. Subunit interactions within the yeast multifactor complex (MFC)**

A number of these multifactor complex associations occur through ionic interactions. Highly acidic stretches in the N-terminal domain (NTD) of eIF3c mediate the binding of eIF1 and eIF5 (Valasek et al., 2002). Conversely, basic domains containing high levels of lysine and arginine residues termed "KR" in eIF1 (Reibarkh et

al., 2008) and "Area II" in eIF5 (Yamamoto et al., 2005) play a critical role in association with eIF3c. Area II in eIF5 also makes critical interactions with the acidic N-terminus tail of eIF1. Similarly, the acidic C-terminus of eIF5 interacts with a basic/hydrophobic surface of eIF1 termed "KH" (Reibarkh et al., 2008). Meanwhile, the AA-boxes (acidic/aromatic) of the eIF5 C-terminal domain have been shown to bind to the lysine rich K-boxes of eIF2β (Asano et al., 1999). Due to the number of ionic interactions involved in multifactor complex (MFC) binding, it is possible that post translational modifications that alter these charges, such as phosphorylation, may play an influential role in MFC formation. Emerging evidence suggests the formation of eIF2/eIF5 complex in mammalian cells may be regulated via phosphorylation (Homma et al., 2005). The mammalian eIF5 C-terminal domain is multiply phosphorylated by CK2 response to cell cycle progression (Homma and Homma, 2005). Expression of eIF5 mutants with alanine substitutions at these residues reduced the formation of eIF5/eIF2/eIF3 complexes both *in vitro* and *in vivo* (Homma et al., 2005). CK2 phosphorylation of eIF5 has been seen in both mammalian and yeast systems. Interestingly, CK2 is also known to phosphorylate mammalian eIF3c and eIF2β subunits and yeast eIF2α (Meggio and Pinna, 2003).

### 1.2. PLANT TRANSLATION

The fundamental processes of translation initiation are similar in all eukaryotes; however, there are some intriguing differences that exist between plants, mammals, and yeast. For the work included in this manuscript plant translation factors have been used exclusively; thus an understanding of the differences between plant factors and those

from other eukaryotes is key when considering the implications. Some of the most notable differences with plant translation from other eukaryotes occur with the eIF4F/eIFiso4F cap-binding complex, eIF2, eIF4B and eIF3 (Browning et al., 2004; Gallie, 2007). When one considers these differences it becomes obvious that plants must use alternative means of regulating protein synthesis during development and in response to environmental signals. The implications of these functional differences on the regulation of plant protein synthesis by protein kinases will be explored further in later sections of this chapter.

The plant translational apparatus is uniquely complicated by the existence of two distinct forms of the cap-binding complex: eIF4F, consisting of eIF4G (p220) and eIF4E (p26), and eIFiso4F, consisting of eIFiso4G (p86) and eIFiso4E (p28; (Browning et al., 1992). eIFiso4F possesses similar functional properties as eIF4F, as both are able to facilitate 5'-cap dependent initiation in an *in vitro* translation system deficient of eIF4F. The cap-binding subunits contain ~50% similarity in their amino acid sequences, while the large subunits contain significantly more differences, sharing only ~35% identity (Browning, 1996). eIFiso4G, which has a molecular mass approximately 100 kDa smaller than eIF4G, lacks a significant amount of sequence in the N-terminal domain. Functional differences in eIF4G and eIFiso4G have been seen in the promotion of internal initiation, cap-independent translation and in the translation of structured mRNA (Gallie and Browning, 2001). As yet the significance of having two forms of the cap-binding complex has not been fully explained; however, it has recently been demonstrated by *in vitro* wheat germ translational assays that mRNAs differentially use eIF4F and eIFiso4F (Mayberry et al, submitted manuscript). While plants possess an

additional cap-binding complex, they appear to altogether lack eIF4E binding proteins (4E-BPs; (Gallie, 2007). By binding to eIF4E, 4E-BPs block the interaction of eIF4E with eIF4G and thus prevent the assembly of the eIF4F complex. 4E-BPs plays a key role in regulating mammalian protein synthesis; however, no obvious homologue has been identified in any plant genome.

The composition of plant eIF3 closely resembles that of mammalian eIF3, having all but one of 13 subunits in common (Burks et al., 2001). Plants appear to lack the key stimulatory subunit (eIF3j) of mammals and yeast (Browning et al., 2001). Unlike mammalian eIF3, which interacts with eIF4B via the eIF3a subunit (Methot et al., 1996), the interaction of plant and yeast eIF4B with eIF3 occurs through the eIF3g subunit (Vornlocher et al., 1999; Park et al., 2004). In light of the critical role eIF3 plays in the assembly of functional initiation complexes and the regulation of translation, these intriguing differences between plant eIF3 and eIF3 from other eukaryotes, particularly *S. cerevisiae,* likely have functional significance for translation or its regulation.

With the exception of plant eIF4B, translation initiation factors are well conserved among eukaryotes at the level of amino acid sequence (Metz et al., 1999). Nevertheless, the function of plant eIF4B is maintained in stabilization of eIF4F binding to 5' cap, PABP binding to the poly(A) tail, and mRNA binding to ribosomes (Cheng and Gallie, 2006). In addition all forms of eIF4B also stimulate the ATP-dependent helicase activity of eIF4A. In mammals, eIF4B, eIF4F, and eIF4A are all necessary for ATP hydrolysis, however in wheat only eIF4A and eIF4F are required (Browning et al., 1989). Although it is not a requisite for ATP hydrolysis activity *in vitro,* wheat eIF4B was found to stimulate activity 2-fold *in vitro* (Browning et al., 1989). In addition, plant eIF4B shares

both a conserved RNA recognition motif and a C-terminal RNA binding domain with other eukaryotes, yet contains a unique N-terminal RNA binding domain (Cheng and Gallie, 2006). This plant-specific binding domain in conjunction with a conserved C-proximal motif is a requisite for the interaction of wheat eIF4B with eIFiso4G (Cheng and Gallie, 2006).

The role plant eIF2 plays in regulating protein synthesis continues to be a source of confusion (Browning, 2004). The eIF2 trimer binds to both GDP and GTP; however eIF2 only associates with Met-tRNA$_i^{Met}$ when complexed with GTP (Gupta et al., 1990). During translation initiation, eIF2 is released from the ribosome following GTP hydrolysis. In order to reform the eIF2 ternary complex (eIF2-GTP-Met-tRNA$_i$), the eIF2 bound GDP must be exchanged for GTP (Erickson and Hannig, 1996). In mammalian systems, the Kd for GDP is 100-fold higher, thus the guanine nucleotide exchange factor eIF2B is required to facilitate the GDP/GTP exchange (Webb and Proud, 1997). However, wheat eIF2 has only a 2-4 fold higher Kd for GTP in the presence of Met-tRNA$_i^{Met}$ (Shaikhin et al., 1992; Benkowski et al., 1995a; Benkowski et al., 1995b), bringing into question the role eIF2B plays, if any, in plant protein synthesis. A plant homlogue of eIF2B has yet to be identified; however, an analysis of the *Arabidopsis thaliana* genome does reveal sequences for all 5 subunits of the eIF2B complex, suggesting that plants may indeed use eIF2B for the recycling of eIF2 (Gallie, 2007).

### 1.3. REGULATION OF TRANSLATION INITIATION BY PHOSPHORYLATION

Protein kinases and phosphatases are integral components of a broad array of signal transduction pathways that are involved in the regulation of transcription,

translation, cell growth, differentiation, and response to an ever-changing extracellular environment. Estimates suggest that some 1000 genes encode protein kinases in the eukaryotic protein kinase superfamily in *A. thaliana,* while another 300 genes encode protein phosphatases (Ichimura et al., 2002). This represents nearly 5% of the *A. thaliana* genome. Protein synthesis is a highly complex pathway with many potential points of regulation; however, most forms of regulation occur with the rate-limiting initiation step. Changes in translational rates have been associated with alterations in the phosphorylation state of a number of factors (Patel et al., 2002); however, the most well characterized mechanisms of regulation involve the phosphorylation of the initiation factors eIF2α and eIF4E-binding proteins (4E-BPs). Interestingly, neither of these pathways appears to play a major role in the regulation of translation initiation in plants, suggesting alternative mechanisms for regulation (Browning, 2004).

Four known kinases phosphorylate mammalian or yeast eIF2α at S51 in response to environmental stress: double stranded RNA-activated protein kinase (PKR), hemin-regulated inhibitor kinase (HRI), PKR-like ER kinase (PERK), and GCN2 (Wek et al., 2006). Since each round of initiation produces eIF2-GDP, GTP/GDP exchange must occur for the initiation cycle to continue. The guanine nucleotide exchange factor eIF2B facilitates this exchange allowing the eIF2 ternary complex to reform. Phosphorylation of the eIF2α subunit on S51 results in it binding more tightly to eIF2B, effectively causing it to be a competitive inhibitor of eIF2B (Wek et al., 2006). As a result, eIF2 GTP/GDP exchange is halted, eIF2 ternary complex formation is limited, and global rates of protein synthesis are reduced. The apparent absence of eIF2B in plants and the markedly reduced affinity of plant eIF2 for GDP initially led to the assumption that plants

do not use phosphorylation of eIF2α as a means of regulating protein translation (Shaikhin et al., 1992). To the contrary however, virally induced plant protein kinase R (pPKR)-like activity has been detected and shown to phosphorylate wheat eIF2α at S51 (Langland et al., 1995). In the presence of double stranded RNA, this kinase was found to inhibit translation *in vitro*, suggesting that plants may use phosphorylation of eIF2α as a means of regulating protein synthesis (Langland et al., 1996). Strangely, a PKR gene sequence has not been identified in a plant genome or expressed sequence tags. Thus, it is not clear that such a kinase functions similarly in plants and thus remains a source of confusion.

For quite some time it has been known that the phosphorylation status of mammalian 4E-BPs, eIF4F, eIF4B and eIF3 are all modulated in response to changes in cellular energy status (Morley and Traugh, 1990); however only recently are the intricacies of the pathways responsible for the regulation of these factors coming to light. Two key kinase cascades involved in regulating the activity of the translation initiation apparatus are the PI3K/mTOR (phosphatidylinositol-3'-kinase/mammalian target of rapamycin) and the MAPK (mitogen activated protein kinase) signaling pathways (Raught and Gingras, 2007). The PI3K/mTOR signaling pathway functions as an energy/redox sensor responsible for the regulation cell growth and proliferation through alterations in protein synthesis (Hay and Sonenberg, 2004). Activation of this pathway by insulin, growth factors, serum, amino acids, and oxidative stress leads to the direct phosphorylation of 4E-BPs and S6 kinase (S6K), which in turn phosphorylates eIF4B and eIF4G (Holz et al., 2005). The activation of S6K, which also phosphorylates ribosomal protein S6, upregulates the translation of mRNAs encoding a number of ribosomal

proteins (Fingar et al., 2004). The MAPK signaling cascade is activated in response to a variety of extracellular stimuli (mitogens) and leads to the regulation of a wide array of cellular processes, including protein translation (Roux and Blenis, 2004). MAP kinases are a linear cascade of three consecutively acting protein kinases, which mediate sequential phosphorylations and are regulated by a web of interactions (Van Drogen and Peter, 2002). Upon activation, MAPK signaling leads to the phosphorylation of 4E-BPs, eIF4E, eIF4B, and PABP (Raught and Gingras, 2007). Through these two pathways the phosphorylation status of several translation initiation factors is regulated to stimulate the translational activity of the cell in response to changes in cellular energy status and mitogenic cues (Raught and Gingras, 2007).

The PI3K/TOR and MAPK pathways in plants have been shown to play a key role in both cell proliferation and stress responses (Jonak et al., 1999; Ichimura et al., 2002; Ingram and Waites, 2006; Deprost et al., 2007). The *A. thaliana* genome encodes a single TOR homologue (AtTOR), and two genes encoding for S6 kinases (Deprost et al., 2007). AtTOR mRNA expression positively correlates with growth, and downregulation via RNA interference halts plant growth and development (Deprost et al., 2007). The *A. thaliana* genome codes for 20 MAP kinases, 10 MAPK kinases, and 60 MAPKK kinases (Ichimura et al., 2002). Yeast and mammals contain three subfamilies of MAP kinases: extracellular signal-related kinases (ERK), stress-activated protein kinases (JNK/SAPK), and p38 kinases (Hog/p38); however, all reported plant MAP kinases appear to belong to the ERK subfamily, and no homologs to the JNK or p38 kinases have been observed (Mishra et al., 2006). In plants MAP kinases appear to play an important roll in signaling for cell division and stress responses (Mishra et al., 2006).

While it is clear that both of these kinase cascades play an important role in plant signaling, the precise impact of each pathway on plant translation has yet to be determined.

Cap binding by the cap-binding protein eIF4E is the rate-limiting step in translation initiation, and as such is a key step for translational regulation (Gingras et al., 1998). The mammalian eIF4E binding proteins (4E-BPs) inhibit cap-dependent translation by preventing the formation of eIF4F, which consists of eIF4E and eIF4G (Beretta et al., 1996). The reversible phosphorylation of 4E-BPs mediates their ability to bind to eIF4E (Gingras et al., 1998). Upon phosphorylation, 4E-BP dissociates from eIF4E, allowing eIF4G access to its binding site and relieving translational inhibition (Gingras et al., 2004). The stimulation of cells with nutrients, growth factors, or hormones stimulates the insulin-signaling cascade, which leads to the phosphorylation of 4E-BP on multiple sites by both PI3K/mTOR and MAPK signaling pathways; it has been demonstrated that activation of either pathway in isolation is sufficient to promote *de novo* translation (Naegele and Morley, 2004). Conversely, nutrient deprivation leads to 4E-BP hypo-phosphorylation and reduced translational rates. Currently, there is no biochemical evidence for 4E-BPs in plants, nor have they been detected in any plant genome (Gallie, 2007). The apparent absence of this factor suggests that plants regulate eIF4F complex formation through some alternative mechanism.

Mammalian eIF4E is phosphorylated by the MAP kinase signal integrating kinase (Mnk) at S209 in response to various extracellular stimuli (Pyronnet et al., 1999). Dephosphorylation of mammalian eIF4E following starvation, mitosis, viral infection, or heat shock is associated with reductions in translational activity (Dyer et al., 2003). It

was initially shown that eIF4E phopsphorylation by Mnk1 lead to an increased cap binding affinity (Minich et al., 1994). Although more recently fluorescence spectroscopy and surface plasmon resonance techniques have been used to demonstrate that phosphorylation of S209 leads to a reduction in the affinity of mammalian eIF4E for capped RNA, suggesting that the improved translational rates are the result of enhanced release of the eIF4 complex prior to AUG recognition (Scheper et al., 2002). The amino acid sequence of eIF4E is generally conserved among eukaryotes, however the site of phosphorylation is poorly conserved between mammalian and plant eIF4E and eIFiso4E (Browning, 2004). However, there are serine and threonine residues in this C-terminal region of both eIF4E and eIFiso4E (Khan and Goss, 2004). Plant eIF4G also lacks the C-terminal region that binds Mnk1, suggesting an alternative kinase may be responsible for the phosphorylation that is seen with plant cap binding proteins (Metz and Browning, 1996). Phosphorylated isoforms of both eIF4E and eIFiso4E have been observed in metabolically active tissues (Browning et al., 2001). In addition, recombinant plant eIFiso4E is reportedly phosphorylated at S207 by mammalian CK2 *in vitro*, leading to a 1.2-2.6 fold reduction in cap binding ability (Khan and Goss, 2004). However, it has been observed by this laboratory that recombinant *A. thaliana* CK2 is unable to phosphorylate either eIF4E or eIFiso4E from wheat.

Both Mnk1 and S6 kinase (S6K) have been shown to phosphorylate mammalian eIF4G resulting in an increase in translational activity (Morley and Traugh, 1990; Pyronnet et al., 1999). The mechanism of this increase in activity was revealed by the chemical cross-linking of eIF4F to cap-labeled mRNA, which showed an increase in the interaction of eIF4E and eIF4G with the 5'-cap of mRNA upon phosphorylation (Morley

and Traugh, 1990). Mammalian eIF4GI contains two clusters of phosphorylation sites, one at the N-terminal third of the protein and a second in the C-terminus of the peptide (Raught et al., 2000; Byrd et al., 2002). Phosphorylation of the C-terminal residues of eIF4G was found to be sensitive to specific inhibitors of the PI3K-mTOR signaling pathway (Raught et al., 2000). Phosphorylation of eIF4G on these residues was determined to yield a "fully active" eIF4G and enhanced formation of active eIF4G-eIF4E complex in cell culture (Raught et al., 2000). Several other protein kinases including PKC, CaMKI (Ca+2/calmodulin-dependent protein kinase I), and PAK2 (p21-activated protein kinase 2) have also been implicated in the phosphorylation of eIF4G (Morley and Traugh, 1990; Qin et al., 2003; Ling et al., 2005).

Treatment of HeLa cells with an inhibitor of MKK-2 (MAP-kinase-kinase-2) leads to a reduction in poly(A) binding protein (PABP) phosphorylation, suggesting PABP phosphorylation is mediated by the MAPK signaling pathway (Ma et al., 2006). A comparative analysis of purified phosphorylated and hypophosphorylated isoforms of wheat PABP has shown that PABP phosphorylation alters the binding to poly(A) RNA and influences interactions with eIF4F, eIFiso4F, and eIF4B (Le et al., 2000). Phosphorylated wheat PABP exhibits cooperative binding to poly(A) RNA, whereas hypophosphorylated PABP binds non-cooperatively (Le et al., 2000). In addition, wheat eIF4B interacted with PABP in a phosphorylation state-specific manner, where phosphorylated eIF4B increased the RNA binding activity of phosphorylated PABP and eIF4F and eIFiso4F stimulated the cooperative binding of hypophosphorylated PABP to RNA (Le et al., 2000).

Serum stimulated eIF4B phosphorylation correlates directly with mammalian cell growth rates and increases in translation rates (Duncan and Hershey, 1987). Large-scale phosphoproteonomics has identified at least 10 unique phosphorylation sites in eIF4B (Raught and Gingras, 2007). Phosphorylation of mammalian eIF4B on S422 by ribosomal S6K plays an important role in mediating the activity of eIF4B, as alanine substitution at this residue renders eIF4B inactive in translation assays *in vitro* (Raught et al., 2004). In addition to S6K phosphorylation, which is mediated by mTOR/PI3K pathway, stimulation of the MAPK pathway also leads to eIF4B phosphorylation at S422 by RSK (p70 Ribosomal S6 Kinase; (Shahbazian et al., 2006). The phosphorylation of eIF4B by S6K or RSK was found to be physiologically significant as it increases interaction of eIF4B with eIF3, leading to increased rates of pre-initiation complex assembly (Shahbazian et al., 2006).

The mammalian eIF3 complex plays a critical role in coordinating the affects of the PI3K/mTOR pathway through direct interactions with both mTOR and its downstream target S6K (Holz et al., 2005). In its inactive state S6K is associated with eIF3 (Peterson and Sabatini, 2005). In response to serum stimulation, mTOR binds to eIF3 complex and phosphorylates S6K1, resulting in its activation and dissociation (Holz et al., 2005). S6K1 is then able to phosphorylate a number of translational targets including eIF4B, which upon phosphorylation associates with eIF3 (Holz et al., 2005). Likely related to this is an increase in the association of eIF4G and eIF3 that has been linked to the mTOR signaling cascade (Harris et al., 2006). The association of eIF3j with the eIF3 core in yeast was also found to be dependent on signaling from the PI3K/mTOR signaling pathway in T-lymphocyte (Miyamoto et al., 2005). Upon

activation of this pathway eIF3j binds to the other eIF3 core subunits, leading to eIF3-40S ribosome association *in vitro*. Interestingly, eIF3j appears to be the only mammalian eIF3 subunit not seen in plants, and it is not clear if the activity of plant eIF3 is regulated by phosphorylation (Burks et al., 2001). Large scale phosphoproteomic analysis of eIF3 has recently identified 29 phosphorylation sites in 8 of the mammalian eIF3 subunits (a, b, c, f, g, h, j, & i; (Damoc et al., 2006).

### 1.4. PROTEIN KINASE CK2

Protein kinase CK2 (formerly known by the misnomer casein kinase II) is a ubiquitous and highly conserved serine/threonine kinase found in both the nucleus and cytoplasm of eukaryotic cells (Pinna, 1997; Litchfield, 2003; Olsten and Litchfield, 2004; Pinna, 2006). CK2 is essential for cell viability, and is involved in key processes such as cell growth and proliferation, transcriptional and translational control, cell cycle progression, and apoptosis. More recently, studies of the molecular clock machinery in *A. thaliana* and *Drosophila* have revealed a role for CK2 in circadian oscillator function (Blau, 2003; Daniel et al., 2004). The lack of an obvious control mechanism, low co-substrate specificity, and the highly acidic nature of CK2 substrates separate it from most other protein kinases.

Initially discovered in 1954 by Burnett and Kennedy, CK2 activity was first described from liver extracts using casein as an artificial substrate (Pinna, 2006). Initial extracts contained both CK1 and CK2 activity, which were distinguished from each other by the end of the 1960s (Pinna, 1994). CK2 was purified to homogeneity in several laboratories and thoroughly characterized (Pinna, 2006). CK2 is now known to

phosphorylate a large number of targets *in vivo*, yet the first physiological target of CK2 (eIF3) was not described until 1976 (Pinna, 1994).

CK2 is likely the most pleiotropic eukaryotic protein kinase, as it is able to phosphorylate more than 300 substrates (Meggio and Pinna, 2003). Of the 307 CK2 substrates recently surveyed, 60 are transcription factors and over 50 regulate DNA/RNA structure and/or play a role in RNA synthesis and translation (Meggio and Pinna, 2003). Over 80 signaling proteins were also identified including 11 calcium binding proteins, 10 protein kinases, and 8 protein phosphatases (Meggio and Pinna, 2003). In addition, over 40 viral proteins have been identified as substrates of CK2, suggesting that its constitutive activity lends itself well to being hijacked by viral machinery. Since such a large number of proteins are targets of CK2 phosphorylation, it has been suggested that it may participate in some global form of cellular regulation, such as the generation of adhesion motifs, caspase targeting, or the destabilization of α-helices (Pinna, 2002). Unlike typical kinases that act in hierarchical signaling cascades, CK2 seems to influence a range of enzymatic processes at various levels (Pinna, 2002).

Regulation of CK2 activity continues to be a source of confusion, as its activity is independent of holoenzyme formation, the presence of second messengers, or phosphorylation events (Litchfield, 2003; Olsten and Litchfield, 2004). CK2 is traditionally viewed as a constitutively active kinase (Olsten and Litchfield, 2004). Unfortunately, it is difficult to reconcile the absence of an "on/off" mechanism with the central role CK2 is believed to play in the regulation of various cellular mechanisms (Meggio and Pinna, 2003). It has been suggested that unlike other kinases, where

regulation is used to trigger activity, regulation of CK2 may be responsible for reducing or fine-tuning activity (Pinna, 2006).

Unlike most protein kinases, CK2 phosphorylates Ser/Thr residues surrounded by multiple acidic residues (Meggio and Pinna, 2003). The consensus phosphorylation motif contains multiple negatively charged side chains downstream from Ser/Thr with particular importance to the n+3 position, and absence of nearby positively charged residues or proline at n+1 (Meggio and Pinna, 2003). An analysis of known CK2 phosphorylation sites has revealed the optimal phosphorylation sequence of CK2 to be EDEE**S**EDEE (Meggio and Pinna, 2003). On average, 5.2 negatively charged side chains (D, E, or another phosphorylated residue) surround each CK2 phosphorylation site (Meggio and Pinna, 2003). A cluster of basic residues ($K^{74}KKKIKREIK^{83}$) that are aligned toward the active site in the crystal structure of CK2 are believed to be responsible for the tendency of CK2 to interact with negatively charged residues (Niefind et al., 1998).

Adding to its uniqueness, CK2 contains a novel co-substrate binding site, which allows it, unlike other protein kinases, to use GTP or ATP as a phosphate donor (Niefind et al., 1999). The crystal structure of CK2α from *Zea mays* first revealed that the purine binding pocket of catalytic subunit lacks residues that are typically required for hydrogen bonding with adenine (Niefind et al., 1998). In ATP binding sites, the adenine moiety is traditionally surrounded by non-polar side chains and fixed in place by three hydrogen bonds; meanwhile in GTP binding sites, hydrogen bonding occurs between the N1-ring nitrogen and an aspartate. In order to bind either ATP or GTP, the CK2 purine binding site is composed of a completely hydrophobic surface that does not allow a specific

hydrogen bonding pattern (Niefind et al., 1999). Interestingly, kinetic analysis of a recombinant maize catalytic subunit revealed $k_{cat}$ values for ATP and GTP that were nearly identical (0.8 $s^{-1}$); meanwhile the holoenzyme values for ATP (4 $s^{-1}$) were nearly twice that of GTP (2 $s^{-1}$; (Riera et al., 2003). This finding suggests co-substrate use may be regulated by the formation of CK2 holoenzymes.

### 1.4.1. CK2 Subunit Composition

The CK2 holoenzyme is a heterotetramer composed of various combinations of two catalytic subunits (α) and two regulatory subunits (β). Reminescent of PKA, the CK2 holoenzyme is formed through dimerization of two regulatory subunits, onto which two non-interacting catalytic subunits are assembled (Boldyreff et al., 1994; Boldyreff et al., 1996; Meggio et al., 2000). β-subunit dimerization occurs prior to any interaction with CK2α, and thus is a prerequisite for formation of the tetrameric holoenzyme. Treatment of CK2β with p-chloromercuribenzoid to modify the four conserved cysteinyl residues prevents dimerization and holoenzyme formation, suggesting that disulfide bonds are playing an important role (Meggio et al., 2000). The traditional view that CK2 forms a stable tetramer is supported by the low dissociation constant (Kd = 5.4 nM) and the ability of recombinant subunits to spontaneously form tetramers *in vitro* (Pinna, 2002). More recently it has become obvious that CK2 subunits do not exist exclusively within the tetrameric holoenzyme, as there is a rapidly growing body of evidence that both CK2α and β exist and perform functions independently of one another (Olsten et al., 2005).

In mammals, the α (42-44 kDa) and α' (38 kDa) subunits, which maintain catalytic activity as monomers, contain a high degree of sequence similarity, although the C-terminal domain of CK2α' is 40 amino acids shorter (Pinna, 1997). Similar α and α' isoforms are seen in yeast. CK2α subunits are highly conserved among different species, suggesting that some of the primary functions would also be conserved. Most kinases are activated by phosphorylation of the T-loop (activation segment), which induces conformational changes that lead to the correct orientation for substrate binding and catalytic group alignment (Johnson et al., 1996). An analysis of the crystal structure of maize CK2α first revealed that CK2 is held in this active conformation by unique interactions of the N-terminal region with both the activation segment and a cluster of basic residues ($K^{74}KKKIKREIK^{83}$) which are responsible for substrate recognition (Niefind et al., 1998). This unique interaction maintains CK2α in an active conformation and is believed to be responsible for the constitutively active character of CK2.

The inappropriate regulation of CK2α subunits has dire consequences on proper cell function. Knockouts of either CK2 catalytic subunit (CKA1, CKA2) in yeast remain viable; however, disruption of both is lethal (Padmanabha et al., 1990). Similar studies in mice indicate that there is some ability of the two isoforms to compensate for one another; however, functional overlap seems incomplete as homozygous CK2α knockout males are sterile and display a defect in spermatogenesis (Xu et al., 1999). In general CK2 levels are high in proliferative tissues; however, elevated expression of CK2α has been associated tumorgenesis, suggesting a link between CK2 and cancer (Landesman-Bollag et al., 2001). The downregulation of CK2 using CK2α siRNA, chemical inhibitors, or antisense CK2α induces apoptosis in cancer cells (Wang et al., 2005).

Meanwhile, antisense expression of the CK2α1 subunit gene in *A. thaliana* implicates CK2 in general plant growth and the regulation of transcription factors for light-regulated genes (Lee et al., 1999a).

In mammalian cells, there is a single β-subunit isoform that forms a homodimer, however there are two distinct isoforms of the yeast regulatory CK2β subunits (Reed et al., 1994). Unlike the catalytic subunits, deletion of the CK2β1 and/or CK2β2 subunits in yeast is not lethal, nor does it appear to affect growth under normal conditions (Reed et al., 1994; Bidwai et al., 1995). All CK2 β-subunits characterized to date contain a zinc finger domain, a short C-terminal positive regulatory domain, and a putative destruction box (Olsten et al., 2005). CK2β subunits share no homology with the regulatory subunits of other protein kinases, with the exception of the *D. melanogaster* Stellate gene product (Riera et al., 2001a). The biological function of Stellate and any relevance to CK2 are unknown. The zinc-finger region of CK2β, which contains 4 cysteine residues, is responsible for β-subunit dimerization (Meggio et al., 2000). Upon dimerization, CK2α subunits associate with the positively charged regulatory domain located at the extreme C-terminus (Niefind et al., 2001). The crystal structure of the CK2 has revealed that the α/β interface is relatively small (832 $Å^2$), suggesting that holoenzyme formation may not be as stable as once predicted (Niefind et al., 2001).

CK2β subunits appear to be ubiquitinated and degraded by a proteasome-dependent pathway (Zhang et al., 2002). CK2β subunits contain a degradation motif often associated with the regulation of cell cyclins. This motif conforms to the general destruction box consensus sequence RxxLxxxxN/D (Allende and Allende, 1995). Appropriately this motif is located on an exposed α-helix, such that it is available for

recognition by the degradation machinery. Since holoenzyme formation modulates CK2 activity, the cell may be able to fine-tune the substrate specificity by regulating the expression of CK2β subunits.

Alone the CK2β subunit has no kinase activity; however, by interacting with CK2α the β-subunit not only confers increased stability, but it will also modulate the interaction and activity toward various substrates (Valero et al., 1997). In the presence of CK2β, the phosphorylation of topoisomerase II and p53 are increased, while the phosphorylation of calmodulin is greatly reduced (Marin et al., 1999; Park et al., 2001). This has lead to the classification of CK2 substrates into one of three categories based on the effect of β subunit (Pinna, 2002)

Over 40 interacting partners of CK2β have been identified; this includes other protein kinases that bind to CK2β in the absence of the catalytic subunit, such as A-Raf, Chk1, Chk2, PKC-ζ, Mos and $p90^{rsk}$ (Bolanos-Garcia et al., 2006). Based on the large number of other proteins that interact with CK2β, it is not unexpected that the CK2β has functions other than regulating the activity of CK2α. Using the yeast-2-hybrid system, the interaction between CK2β and the MAP kinase pathway relay protein (A-Raf) was first identified (Boldyreff and Issinger, 1997). The binding site was later mapped to residues 255-569 which align with the CK2α-binding domain. Upon binding to CK2β, A-Raf kinase activity toward MEK is enhanced 10-fold (Hagemann et al., 1997). This activation was prevented by the addition of the CK2α subunit.

### 1.4.2. Plant CK2

In plants, CK2 activity was first described in the early 1980s by Yan and Tao, who purified and characterized monomeric CK2α from wheat germ (Yan and Tao, 1982a). Since this discovery, CK2 has been studied in a variety of other plants including maize (Riera et al., 2001b), tobacco (Salinas et al., 2001), *A. thaliana* (Sugano et al., 1998), and mustard (Ogrzewalla et al., 2002) in various oligomeric forms. Although a much lower number (<30) of *in vitro* substrates have been described in plants, it is evident that CK2 is involved in several unique plant processes, such as light signaling, circadian rhythm, seed storage, and salic-acid mediated defense (Salinas et al., 2006).

The *A. thalania* genome encodes four distinct catalytic subunits, one of which contains a unique N-terminal chloroplast designation peptide. A comparison of *A. thaliana* CK2α sequences with those from human reveals a high level of identity (82-90%), with all key structural determinants conserved in all subunits (Salinas et al., 2006). Plant CK2α is approximately 60 amino acids longer than mammalian CK2α, due to truncation of the C-terminal domain. There is some speculation that the truncated C-terminal domain of plant CK2α is responsible for the increased stability and higher specific activity of monomeric plant CK2α subunit when compared to monomeric mammalian CK2α (Riera et al., 2001b).

In plants, the number of genes encoding CK2β-subunits is more extensive than in other systems described to date. The mammalian genome encodes a single isoform of CK2β, which forms a homo-dimer prior to the binding of catalytic α subunits (Boldyreff et al., 1996). Two CK2β genes have been identified in yeast, and recent data indicates

that the formation of a β1β2 dimer is necessary for yeast holoenzyme formation (Kubinski et al., 2007). Thus both mammals and yeast only posses a single version of the CK2β dimer, β1β1 in mammals and β1β2 in yeast. Plants, however, posses multiple isoforms of CK2β subunits and, at least some isoforms appear to be capable of forming both homo- and hetero-dimers *in vitro* (Riera et al., 2001b; Riera et al., 2003).

The expression and subcellular localization of four distinct β-subunits in *A. thaliana* was recently described (Salinas et al., 2006). *A. thaliana* CK2β subunits have a high degree of identity among themselves, however when compared to the regulatory subunits of other organisms they are far less conserved than catalytic subunits. Although the *A. thaliana* subunits contain the general features of other CK2β subunits (CK2 regulatory signature, degradation motif, & cysteine-rich zinc finger), there is a plant specific N-terminal extension of unknown function (Salinas et al., 2006). This region contains several residues believed to be plant specific autophosphorylation sites in addition to the autophosphorylation domain plant CK2β shares with β-subunits from other systems.

An analysis of the subcellular localization of the eight CK2 α and β subunits in *A. thaliana* revealed that while some functional overlap is likely, subunit specialization also exists (Salinas et al., 2006). Genomic sequences surrounding the CK2 genes of *A. thaliana* suggest that αA & αB, β1 & β2, and β3 & β4 occur on two self-duplicated regions of the genome. Surprisingly, the expression of CK2 genes as green fluorescent protein fusions revealed that β1 and β3 localize to the cytosol and nucleus, while β2 localizes exclusively to the nucleus and β4 to the cytosol (Salinas et al., 2006). It is suspected that after duplication, certain genes evolved to code for proteins with different

subcellular specializations and differential substrate specificities (Salinas et al., 2006); however, this has yet to be proven. This theory is supported by work in maize that has revealed the differential expression of three maize CK2β subunits during development (Riera et al., 2001b) and the plant cell cycle (Espunya et al., 2005).

### 1.5. CK2 PHOSPHORYLATION AND PROTEIN SYNTHESIS

A number of *in vivo* CK2 phosphorylation sites have been identified in translation initiation factors in yeast and mammalian cells (Meggio and Pinna, 2003); however, the exact role CK2 phosphorylation plays in translation is not known. CK2 phosphorylation sites in mammalian eIF2B, eIF2β, and eIF5 appear to be necessary for the proper regulation of translation (Wang et al., 2001; Homma et al., 2005; Llorens et al., 2005a). The phosphorylation of mammalian eIF2β by CK2 leads to a reduction in its affinity for GDP (Singh et al., 1994), and alanine substitution to prevent CK2 phosphorylation results in reduced rates of protein synthesis (Llorens et al., 2005a). Meanwhile, phosphorylation of mammalian eIF2B on its epsilon subunit by CK2 is required for interaction of the guanine nucleotide exchange factor with eIF2 *in vivo* (Wang et al., 2001). In both of these instances, CK2 phosphorylation would appear to enhance the recycling of eIF2 and improve ternary complex formation. Similarly, alanine substitution at CK2 phosphorylation sites in mammalian eIF5 limits the formation of mature multifactor complexes, leading to a disruption in synchronous cell progression and reduced growth rates (Homma et al., 2005). Additional studies have implicated CK2 in the phosphorylation of eIF4E, eIF4B, and eIF2α (Meggio and Pinna, 2003); however, the effect phosphorylation has on these factors is less clear. In mammalian cells, CK2 has

also been found to phosphorylate and upregulate the activity of Akt/PKB, suggesting a potential role for the kinase in regulating translation initiation via the mTOR signaling pathway (Di Maira et al., 2005).

Far less is known about the role CK2 plays in regulating plant translation initiation. Plants possess translational machinery that does not appear to be regulated through the classical phosphorylation pathways seen in mammals and yeast. Yet, a number of plant initiation factors are phosphoproteins (Browning, 2004), and changes in phosphorylation states of plant factors have been linked to the interaction of eIF4B with PABP and the formation of cap-binding complexes (Le et al., 2000; Khan and Goss, 2004). All plant genomes analyzed to date appear to encode multiple CK2 subunits, and thus posses the potential for forming a diverse range of CK2 kinase holoenzymes in response to changes in cell physiology. With their enhanced set of CK2 isofoms, plants may be able to regulate translation initiation through alterations in the phosphorylation status of various initiation factors. The studies included in this dissertation represent a starting point for evaluating the role CK2 phosphorylation plays in regulating plant translation initiation.

# Chapter 2: Differential Phosphorylation of Plant Initiation Factors by Recombinant *Arabidopsis thaliana* CK2 Holoenzymes

## 2.1. INTRODUCTION

Protein kinase CK2 (formerly known as casein kinase II) is a ubiquitous and highly conserved serine/threonine kinase found in both the nucleus and cytoplasm of all eukaryotic cells (Pinna, 2002; Meggio and Pinna, 2003; Litchfield, 2003; Olsten and Litchfield, 2004; Olsten et al., 2005; Pinna, 2006). CK2 is essential for cell viability, and is involved in processes such as cell proliferation, transcriptional and translational control, cell cycle progression, and apoptosis (Litchfield, 2003). The lack of an obvious control mechanism, low co-substrate specificity (both ATP and GTP can be used as phosphate donors), and the acidophillic nature of CK2 separate it biochemically from most other protein kinases (Pinna, 2002). CK2 has been found to phosphorylate over 300 substrates *in vivo*, including 60 transcription factors and over 50 proteins that regulate DNA/RNA structure and/or play a role in RNA synthesis and translation (Meggio and Pinna, 2003).

CK2 has a tetrameric structure consisting of two catalytic α-subunits that assemble with two regulatory β-subunits to form a tetrameric holoenzyme (Boldyreff et al., 1994; Boldyreff et al., 1996; Graham and Litchfield, 2000). In mammalian cells, there is a single β-subunit isoform that forms a homodimer, onto which two catalytic subunits assemble (α/α, α′/α′, or α/α′). Yeast also posses two distinct catalytic subunits (α and α′), however there are also two distinct isoforms of the yeast regulatory CK2β subunits (Reed et al., 1994). In yeast, both the CK2β and CK2β′ subunits are required

for formation of the β-β´-dimer (Kubinski et al., 2007). Thus, there are three potential forms of CK2 holoenzymes in yeast and mammalian systems. Plants however contain a number of isoforms for both catalytic and regulatory subunits (Riera et al., 2001b), creating the potential for a wider variety of CK2 holoenzyme combinations.

The increased number of CK2 isoforms in plants presents a novel mechanism for regulating CK2 activity. The catalytic activity of mammalian and yeast CK2 holoenzymes toward various substrates is generally higher than with catalytic subunits alone (Sarno et al., 2002). However, some substrates are only phosphorylated in the presence of the CK2 holoenzyme, while others are only phosphorylated by the catalytic monomer (Benaim and Villalobo, 2002; Pinna, 2003). It is not currently known if various isoforms of plant CK2β subunits confer substrate specificity; however, by regulating the levels of specific CK2β subunits plants may be able to fine-tune CK2 phosphorylation.

A systematic study of the *Arabidopsis thaliana* genome recently identified four genes coding for CK2α subunits and four genes coding for CK2β subunits (Salinas et al., 2006). Using transgenic plants and agroinfiltration, it was revealed that these CK2β regulatory subunit isoforms were found to exhibit a diverse subcellular distribution (Salinas et al., 2006). Additionally, work in maize has revealed the differential expression of three maize CK2β subunits during development (Riera et al., 2001b) and the plant cell cycle (Espunya et al., 2005). Yeast-two hybrid data obtained from various maize CK2β subunits predicts that three CK2β subunits are able to interact *in vivo* with one another (Riera et al., 2001b), and at least some isoforms of recombinant maize CK2β subunits have been shown to form homodimers *in vitro* (Riera et al., 2001b; Riera et al.,

2003). However, it is not known if this is the case for all isoforms of the plant β-subunits. Interestingly, yeast-2-hybrid results from maize CK2β2 also suggest it was fully unable to interact with itself, and it was suspected that this was the result of a $Val^{212}$ to $Ala^{212}$ substitution in the cysteine-rich dimerization domain (Riera et al., 2001b). No such substitution exists in *A. thaliana* CK2β subunits. Since plants possess multiple isoforms of the CK2 subunits, regulation of CK2β subunit expression and variations in the subcellular distribution of the CK2β isoforms presents a potential mechanism for regulating the substrate specificity of CK2 through the formation of different CK2 holoenzyme complexes.

CK2β subunits appear to be ubiquitinated and degraded by a proteasome-dependent pathway (Zhang et al., 2002). All CK2β subunits characterized to date contain a degradation motif that is associated with the regulation of cell cyclins, and conforms to the general destruction box consensus sequence RxxLxxxxN/D (Allende and Allende, 1995). Appropriately this motif is located on an exposed α-helix, such that it is available for recognition by degradation machinery. Phosphorylation of *A. thaliana* CK2β4 leads to ubiquitination and degradation by the proteasome pathway in a manner that is regulated by the circadian clock (Perales et al., 2006). By regulating the expression and degradation of CK2β subunits, the cell may be able to fine-tune the specificity of CK2 in response to the cell cycle or circadian clock.

A protein kinase isolated from wheat germ was shown to phosphorylate an unknown "target" protein and various plant initiation factors (Yan and Tao, 1982a; Yan and Tao, 1982b; Browning et al., 1985). While characterization of the kinase revealed casein-kinase like properties, the kinase's specific identity was unknown. Using mass

spectrometry, the study presented here unambiguously identifies the previously described protein kinase as CK2. In this work, two of the four CK2α subunits and all four CK2β subunits from *A. thaliana* were cloned and expressed in *E. coli*. The ability of these recombinant enzymes to form stable holoenzymes, and their capacity to phosphorylate various plant translation initiation factors both as holoenzymes and monomers was characterized in this study. All four recombinant *A. thaliana* CK2β subunits spontaneously dimerized, assembled into holoenzymes, and exhibited autophosphorylation in the presence of catalytic subunits. In addition, it has been shown that CK2 not only phosphorylates plant eIF2α and eIF3c, but various forms of recombinant CK2 are capable of differentially phosphorylating plant eIF2β, eIF4B, and eIF5. The results presented here suggest that CK2 may play an important role in regulating translation initiation in plants.

## 2.2. METHODS

### 2.2.1. Materials

All chemicals were of high quality and obtained from Sigma, Fisher, VWR or as indicated. Chromatography resins used were DEAE-sepharose (Whatman) phosphocellulose P11 (Whatman), Sephacryl S-200 HR (GE Biosciences), and Ni-NTA Superflow (Qiagen). The buffers used for chromatography were Buffer B (20 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and KCl as indicated, Buffer B-100 is Buffer B containing 100 mM KCl), Buffer C (50 mM HEPES-KOH, pH 7.6, 600 mM KCl, 20 mM imidazole) and Buffer D (20 mM HEPES-KOH, pH 7.6, 250 mM imidazole, and KCl as indicated).

### 2.2.2. Purification of Wheat Germ Casein Kinase

Wheat germ S30 was prepared from wheat germ (200g) as previously described (Lax et al., 1986; McInerney et al., 2006). Wheat germ kinase was purified from S30 using DEAE-cellulose and phosphocellulose as previously described (Yan and Tao, 1982a). Phosphocellulose elution fractions containing peak activity (8 ml containing 9.3 mg of protein) were pooled and concentrated to approximately 1 ml using an Amicon Ultra-4 centrifugal filter (Millipore). The sample was then loaded onto a 126 ml Sephacryl S-200 HR column (GE Biosciences). A small amount of activity (~15%) eluted in the column's void volume, indicating a MW greater than 200 kDa. It is likely that this fraction represented CK2 bound to other protein complexes or oligomers of CK2 subunits, as the expected molecular weight of the CK2 holoenzyme is ~146 kDa. The majority of the kinase activity eluted as a monomer (~37 kDa). Three fractions with peak kinase activity were pooled and concentrated by centrifugal filtration to a final volume of 200 µl. The purified kinase was divided into 25 µl aliquots, quick frozen in dry ice and stored at –80°C for further analysis.

### 2.2.3. General Kinase Assay

The incubation mixture (20 µl) contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~250 cpm/pmol), 1-3 µg of the indicated substrate (1 µg histone deacetylase 2β (HD2β) or 3 µg eIF3) and 2-5 µl of the fraction containing kinase activity. Incubation was at 30 $^{o}$C for 20 min. The reaction was terminated by the addition of 4x SDS loading dye (2.5 M Tris, pH 6.8, 40% glycerol, 8%

SDS, 0.1% bromophenol blue). Samples were then heated at 100°C for 5 min and loaded on a 12.5% SDS-PAGE. Following electrophoresis, gels were dried, and exposed to a phosphorimager screen (Molecular Dynamics) for 2-12 hours. The screen was then analyzed using Molecular Imager FX and Quantity One Software (BioRad).

### 2.2.4. Identification of Wheat Germ Casein Kinase

To identify the protein responsible for the kinase activity, 20 µl of the concentrated sample was run on a 12.5% Bis-Tris gel using MOPS running buffer (Invitrogen). Upon staining with colloidal coomassie blue (Invitrogen), the sample was determined to contain five major proteins. Each of these bands was cut from the gel and destained using 500 µl deionized water. Each gel slice was dehydrated with 200 µl acetonitrile (Sigma). The dehydration process was repeated until each gel slice turned white and was air-dried for 10 min. Each sample was reduced with 10 µl of 100 mM DTT for 30 min, and alkylated with 100 µl of 100 mM iodoacetamide for 30 min. The samples were washed with 200 µl of 100 mM ammonium bicarbonate, before repeating the dehydration process. Trypsin (0.2 µg, Promega) suspended in 20 µl of 50 mM ammonium bicarbonate was added to each gel sample and incubated overnight at 37$^{o}$C. Following incubation, 40 µl of 5% formic acid in 100 mM ammonium bicarbonate was used to extract peptides from each sample.

Peptide identification was performed in the Analytical Instrumentation Facility Core (Dr. Maria Person, Director) using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems) as previously described (Shen et al., 2007).

Briefly, each tryptic digest was dried to <5 µl in a SpeedVac (ThermoSavant), desalted with a Ziptip µ-C18 pipet tip (Millipore), and eluted with 1.5 µl of matrix onto three spots on the MALDI target. A solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid diluted in half with a solvent was used to saturate the matrix. Calibration Mixture 4700 (Applied Biosciences) was prepared according to manufacturer's instructions.

Spectra were acquired automatically using a 4000 Series Explorer V3.0 RC1. MS spectra were obtained using 2000 laser shots over a mass range of 700-4000 and calibrated internally on trypsin autolysis peaks. The top 20 MS signals with minimum S/N 20 were selected for MS/MS fragmentation after exclusion of matrix, trypsin, and keratin peaks. GPS Explorer v3.5 was used to perform additional peak processing and a database search. Additional searches were performed with MASCOT V2.0 (http://www.matrixscience.com). Samples were searched against Swiss-Prot, Trembl, and EST databases.

### 2.2.5. Cloning and Expression of *A. thaliana* CK2 Subunits

Approximately 500 mg of *A. thaliana* flowers were ground to a powder in liquid nitrogen and homogenized in 4 ml of TRIzol reagent (Invitrogen). RNA isolation was performed as described by the manufacturer. Total RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) in a 25 µl RT-PCR reaction using 5 µg RNA, 40 U RNAseOUT (Invitrogen), dNTPs (0.5 mM each), 10 mM DTT, and 20 µg/ml oligo dT. Full length *A. thaliana* CK2 subunit cDNAs were amplified in a thermal-cycler

as follows: 10 min at 95 °C; 1 min at 94 °C; 1 min at 55 °C; 1.5 min at 72 °C for 30 cycles, followed by 10 min at 72 °C. Each 50 µl reaction contained 2.5 U FastStart High Fidelity Enzyme Blend (Roche), 5 µl FastStart Reaction Buffer, 200 µM dNTP mix, 100 ng of first strand RNA, and 10 pmol of each primer.

For bacterial expression, appropriate primers were designed for cloning into pET23d (Novagen), such that each subunit could be expressed with a C-terminal 6xHis-tag. PCR products from CK2 subunits α1 (At5g67380), α2 (At3g50000), β1 (At5g47080), β2 (At4g17640), β3 (At3g60250), and β4 (At2g44680) were cloned into pET23d(+) vector using *NcoI/XhoI* restriction sites. All constructs were confirmed by DNA sequencing (DNA Core Facility, Institute for Cell and Molecular Biology, University of Texas). For a list of primers used see Table 2.2 in Appendix.

Each CK2-pET23d subunit construct was transformed into BL21(DE3) *E. coli* cells for recombinant protein expression. Overnight cultures (50 ml LB media containing 50 µg ampicillin) started from a single colony were used to inoculate 2 x 1L of LB media (Invitrogen) and grown to an $A_{600}$ of 0.5 at 37°C. Expression of protein was induced with the addition of IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 0.6 mM. Following IPTG induction, cells were grown for 3 hr at 30°C. Cells were harvested by centrifugation (6000 x *g*) for 15 min at 4°C. Each *E. coli* cell pellet was resuspended in 30 ml Buffer C and 1 small Complete protease mini inhibitor tablet (EDTA-free, Roche). The cells were disrupted by sonication for 3 X 30 sec at 70% power and 2 x 90% power using a Vibra Cell sonicator (Sonics & Materials Inc.). Lysed cells were centrifuged at 184,048 x *g* for 30 min at 4°C.

### 2.2.6. Purification of 6xHis-tag Proteins

Cell lysates were filtered through a 0.2 μm filter (Millipore) and loaded onto a 2 ml Ni-NTA column equilibrated in Buffer C. The column was washed with Buffer C until the $A_{280}$ returned to baseline. 6xHis-tagged proteins were then eluted from the column with Buffer D containing 300 mM KCl for CK2α or 100 mM KCl for CK2β subunits. Fractions of 1 ml were collected and analyzed by a Bradford protein assay. Those containing the highest amount of protein were immediately applied to a 1 ml phosphocellulose P11 column equilibrated in either Buffer B-300 or Buffer B-100, for α and β subunits respectively. For CK2α subunits, the proteins were then eluted with a 10X gradient (10 ml) of Buffer B containing 0.3 to 1 M KCl. CK2β subunits were eluted with a 10X gradient (10 ml) of Buffer B containing 0.1 to 0.5 M KCl. All fractions were analyzed by SDS-PAGE, and those containing the highest purity and concentration were pooled. CK2α fractions were dialyzed overnight at 4$^{o}$C in Buffer B-100. CK2β subunits were not dialyzed as this resulted in a significant loss of protein, presumably by binding to the dialysis tubing.

### 2.2.7. Reconstitution of CK2 Holoenzymes *in vitro*

CK2α (300 μg) was applied to a FPLC HiPrep 16/60 Sephacryl S-200 HR column (126 ml bed volume, GE Biosciences) equilibrated in Buffer B-180 and the elution was monitored by $A_{280}$ for 120 ml. CK2β (300 μg) and the CK2αβ holoenzymes were analyzed in a similar manner. To analyze holoenzyme formation using gel filtration, ~300 μg of CK2α1 and CK2β (β1, β2, β3, or β4) were combined in a 1:1 molar ratio (164

μg CK2α1 and 135 μg CK2β), the KCl concentration was adjusted to 180 mM using Buffer B, and incubated on ice for 1 hour prior application to the column, and the elution was monitored by $A_{280}$.

To analyze the phosphorylation of initiation factors by CK2 holoenzymes, eight holoenzyme complexes were formed *in vitro* (CK2α1β1, CK2α1β2, CK2α1β3, CK2α1β4, CK2α2β1, CK2α2β2, CK2α2β3, CK2α2β4). A buffer exchange to Buffer B-100 was performed on each CK2β subunit using 1 ml of G-25 (Whatman). Eight 50 μl reactions each contained 250 pmoles of total CK2 holoenzyme (tetramer of 2 CK2α subunits and 2 CK2β subunits; 500 pmol of CK2α and 500 pmoles of CK2β) and a final KCl concentration of 50 mM in Buffer B, were incubated on ice for 1 hr. For each reaction, 20 μg of either CK2α1 or CK2α2 was combined with 16.5 μg of CK2β1, β2, β3, or β4. To verify complex formation, 12 μl of each reaction was run on a 4-20% Tris-Glycine non-denaturing gel (Invitrogen). Each of these holoenzyme preparations was used to evaluate the activity toward various substrates.

### 2.2.8. Identification of T-substrate

A 48 kDa protein termed T-substrate was previously identified as an endogenous phosphoryl acceptor for the wheat germ kinase used in this study (Yan and Tao, 1982a). T-substrate was purified from wheat germ and analyzed by mass spectrometry as described above for the kinase.

### 2.2.9. Cloning, Expression, and Purification of CK2 Substrates

To obtain non-phosphorylated CK2 substrates for analysis, wheat initiation factors and HD2β were cloned and expressed in *E. coli*. Briefly, the coding region for each target was PCR amplified from an expressed sequence tag (EST), cloned into a pET vector (Novagen) for bacterial expression, and purified using either phosphocellulose or a Ni-NTA matrix. All primers used are listed in Table 1.2 in Appendix.

*Wheat eIF4B, and eIF5.* The cloning, expression, and purification of eIF4B and eIF5 has been previously described (Mayberry et al., 2007).

*Wheat eIF2.* Wheat eIF2α (p42) was previously cloned into pET30a(+) for bacterial expression (Chang et al., 1999). The eIF2α cDNA was amplified from this construct and cloned into the *NdeI/BamHI* site of pET15b(+). This new eIF2α-6xHis construct was transformed into BL21(DE3) *E. coli* cells and expressed and purified as described above for CK2β subunits. Wheat eIF2β (p38) was prepared as previously described (Metz and Browning, 1997).

*Wheat eIF3c.* The cDNA for eIF3c was amplified from expressed sequence tag *BJ257961* and cloned into pET23d (+). This construct adds a C-terminal 6xHis tag when using the *NcoI/XhoI* site. The plasmid was transformed into Arctic Express DE3(RIL) *E. coli* cells (Stratagene). A 50 ml culture was inoculated with a single colony and grown overnight at 37°C. The overnight culture was used to inoculate 2 x 1 L of LB. Cells were grown at 25° C to an $A_{600}$ of 0.5, induced with 0.6 mM IPTG, and allowed to grow for 3 hr at 25°C. The cells were harvested and His-eIF3c was purified as previously described for CK2β subunits.

*T-substrate/HD2β.* Primers were designed for cloning *A. thaliana* HD2β cDNA into the pET23d(+) expression vector with a C-terminal 6x-His-tag using *NcoI/XhoI* as described above. The HD2β-6xHis construct was transformed into BL21(DE3) *E. coli* cells and expressed and purified as described above for CK2β subunits.

### 2.2.10. Phosphorylation of CK2 Substrates

For translation initiation factor phosphorylation, a kinase assay similar to the one described above was conducted using ~3 μg of purified translation initiation factor as substrate. Generally, the incubation mixture contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~250 cpm/pmol), and 100 mM KCl. Reactions were run on 12.5% SDS PAGE and a phosphorimage was obtained as previously described.

To compare the activity of the various forms of recombinant CK2, approximately 1 μg of each substrate was incubated with 0.5 pmol of CK2α or 0.25 pmol of holoenzyme (tetramer containing 0.5 pmol CK2α + 0.5 pmol CK2β) in reactions containing 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~250 cpm/pmol), and 100 mM KCl at 25°C for 15 min. Each reaction was stopped by the addition of 4x SDS loading dye. The samples were boiled for 5 min and analyzed by SDS-PAGE. The phosphorylation of each substrate was visualized by phosphorimaging. Quantitative densitometry was performed using Quantity One (BioRad) and results were expressed as counts/mm$^2$. Each substrate was run in triplicate for each of the 10 recombinant CK2

kinases (CK2α1 only, CK2α2 only, CK2α1β1, CK2α1β2, CK2α1β3, CK2α1β4, CK2α2β1, CK2α2β2, CK2α2β3, CK2α2β4).

To quantify the number of phosphorylation sites present in each substrate, the phosphorylation reaction was carried out in 50 µl containing 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~100 cpm/pmol), and 100 mM KCl, 10 µg of substrate, 5 pmol of recombinant CK2α1, and an additional 5 µg of acetylated BSA. Reactions were incubated at 30°C for 5, 15, or 30 min, stopped by the addition of 1 ml HMK buffer (20 mM Hepes/KOH, pH 7.6, 2.5 mM MgCl2, and 100 mM KCl) and immediately passed through a nitrocellulose filter (MFS, 0.45 um) that had been pre-soaked in 1 mM $KPO_4$, pH 6.8. Each filter was washed with approximately 2 mL of cold HMK buffer, dried, and analyzed using a scintillation counter. Each value was corrected for the amount of [$^{32}$P] retained by the filter in the absence of substrate. CK2β appears to be a requisite for phosphorylation of eIF2β; therefore, the CK2α1β1 holoenzyme was used in place of CK2α1 alone for its phosphorylation. For this reaction, a CK2α1β1 blank was run to account for autophosphorylation of the CK2β subunit.

## 2.3. RESULTS AND DISCUSSION

### 2.3.1 Purification of Native CK2

The wheat germ kinase previously shown to phosphorylate eIF3c and eIF2α (Browning et al., 1985) was identified as casein kinase II alpha (CK2α) using mass spectrometry. The S-200 HR gel filtration analysis and evaluation of kinase specific activity in elution fractions is included in Appendix Figure 2.10. Mascot search resuts

identied a high probability match with gi|14317906|dbj|BAB59136.1. This identification represents 17 matched peptides with 31% sequence coverage (see Appendix Figure 2.11). The CK2α purified from wheat germ (Figure 2.1, band 1) eluted from Sephacryl S-200 HR with several other proteins that were identified as 60 S ribosomal protein S7 (28 kDa; band 3), 40S ribosomal protein S19 (19 kDa; band 4), and two isoforms of 60S ribosomal protein L3 (16/17 kDa; bands 5 a,b). Recently an extensive analysis identified interacting partners of mammalian 40S ribosomal protein S19 (RPS19). RPS19 was found to interact with mammalian orthologs of three of the plant proteins identified in our sample: 60S ribosomal protein L3, 60S ribosomal protein S7, and CK2α subunits (Orru et al., 2007). It is possible that CK2α associates with these ribosomal proteins and is recruited to phosphorylate various initiation factors bound to ribosomes or other ribosomal proteins that are known to be substrates (Abramczyk et al., 2003). There is some speculation that CK2 phosphorylation could regulate ribosome assembly or the degradation of specific proteins (Nusspaumer et al., 2000; Szebeni et al., 2003; De Marchis et al., 2005).

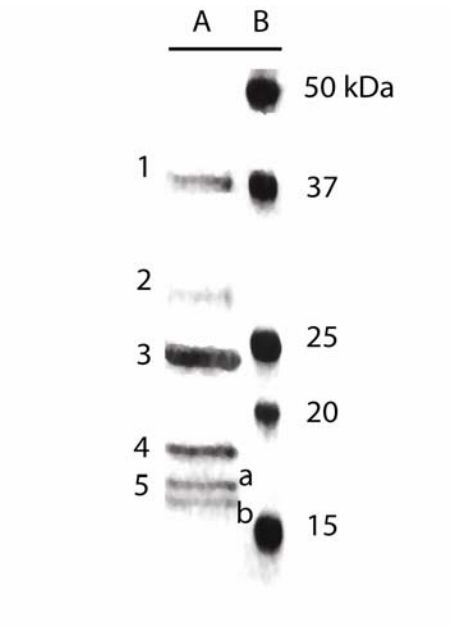



**Figure 2.1. Native wheat CK2 preparation.**

Silver stained SDS-PAGE of the Sephacryl S-200 HR protein preparation (Lane A) and BioRad Precision Plus molecular weight markers (Lane B). The native wheat germ kinase (band 1) was identified as CK2 by mass spectrometry. See Appendix Figure 2.11 for MS analysis of band 1. The S-200 column elution contains an unidentified peptide (band 2) and several smaller ribosomal proteins: ribosomal S7 (band 3, 24 kDa), 40S ribosomal protein S19 (band 4, 19 kDa), and the A and B isoforms of 60S ribosomal protein L3 (bands 5, a/b; 16/17 kDa, respectively).

### 2.3.2. Expression & Purification of *A. thaliana* CK2 Subunits

cDNAs for 2 catalytic and 4 regulatory *A. thaliana* CK2 subunits were obtained by reverse transcription of mRNA and cloned into pET23d(+) with a 6xHis-tag. A single variation was detected in the cDNA for CK2β4, where an alanine insertion occurred after amino acid residue 240 due to the inclusion of the first three nucleotides (GCA) after the predicted splicing site of the fourth intron. Approximately half of all expressed sequence tags for CK2β4 in Genbank contain this variation, suggesting alternate splicing at this site. *A. thaliana* CK2 subunits were overexpressed separately in *E. coli* and purified to characterize their biochemical properties. A high amount of soluble CK2 subunit was obtained for all six subunits (2-10 mg) following purification on Ni-NTA resin; however, a small amount of contaminant protein was present in the imidazole eluant. The protein was further purified and the imidazole was removed by chromatography on phosphocellulose. Six recombinant *A. thaliana* CK2 subunits (α1, α2, β1, β2, β3, & β4) were purified to near homogeneity as shown in Figure 2.2. Attempts to amplify CK2α3 cDNA (At2g2308) from total flower RNA were unsuccessful. CK2αcp (At2g23070) was successfully cloned, overexpressed in *E. coli*, and initial attempts were made at purification (result not shown). However, due to the chloroplast specific designation of this subunit it was not pursued for further studies.

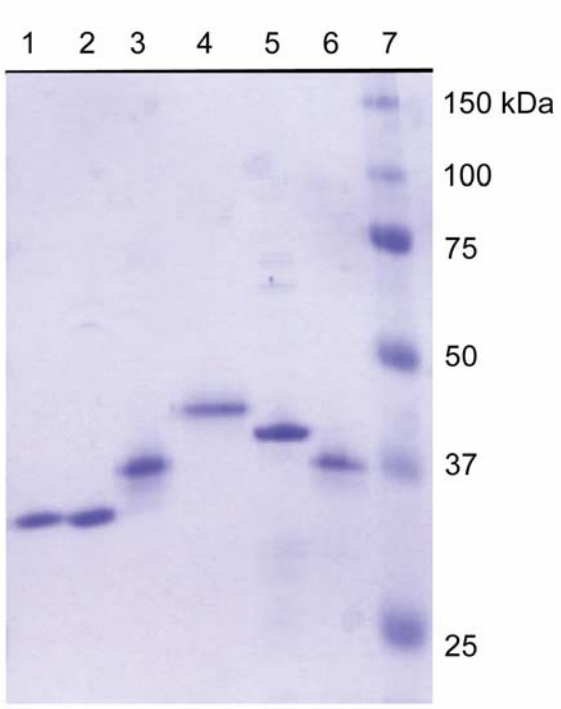



**Figure 2.2. SDS-PAGE analysis of recombinant *A. thaliana* CK2 subunits**

Each lane contains 1.5 µg of each purified recombinant *A. thaliana* CK2 subunit. Lane 1, CK2α1; Lane 2, CK2α2; Lane 3, CK2β1; Lane 4, CK2β2; Lane 5 CK2β3; Lane 6, CK2β4; Lane 7. Precision protein standards (Bio-Rad) as indicated.

### 2.3.3. Formation of Tetrameric Holoenzyme Complexes

The ability of recombinant *A. thaliana* protein kinase subunits CK2α1, CK2α2, CK2β1, CK2β2, CK2β3, or CK2β4 to form tetrameric holoenzyme complexes *in vitro* was evaluated by FPLC gel filtration using a Sephacryl S-200 HR column. A summary of the results is shown in Table 2.1. CK2α1 (40 kDa) elutes from Sephacryl-200 HR at

63.2 ml between the ovalbumin (59.7 ml, 43 kDa) and chymotrypsin (73 ml, 25 kDa) as expected (see Figure 2.3). CK2β1 elutes from S-200 HR at 52.9 ml, corresponding to a molecular weight of ~70 kDa. This suggests that the recombinant plant CK2β subunit is forming a dimer, since the predicted molecular weight of the monomeric β1 subunit is 33.5 kDa. Similar results were obtained for all four CK2β subunits, suggesting recombinant *A. thaliana* CK2β subunits spontaneously form homodimers *in vitro* (Table 2.1). When combined in a 1:1 molar ratio, the CK2α1 and CK2β1 subunit elution profiles shifts to a single higher molecular weight peak (Figure 2.3). The peak of this CK2 holoenzyme product elutes from S-200 HR just after aldolase (152 kDa) at 44.4 ml, suggesting a molecular weight of ~127 kDa. The predicted molecular weight of the tetrameric holoenzyme, which consists of a β1 dimer and two α1 subunits is 147.5 kDa. Based on the elution profile, it appears that the α1 and β1 subunits form holoenzyme complexes when combined in a 1:1 molar ratio. Interestingly in the absence of catalytic subunit, a small number of CK2β subunits seem to form higher order aggregates, as indicated by a small absorbance peak that occurs in the column's void volume. It is also possible that this higher molecular weight absorbance represents contaminants; however, none are visible in SDS-PAGE analysis (Figure 2.2). Similar results were obtained with other *A. thaliana* CK2β regulatory subunits when combined with CK2α1 (see Table 2.1).

**Table 2.1. FPLC Sephacryl S-200 gel filtration of CK2 subunits & holoenzymes**

| Samples | Predicted Mass (kDa) | Elution Volume (ml) | Observed Mass (kDa) |
|---|---|---|---|
| Aldolase | 152 | 41.3 | |
| Albumin | 67 | 53.9 | |
| Ovalbumin | 43 | 59.7 | |
| Chymotrypsin | 25 | 73 | |
| | | | |
| CK2α1 | 40.3 | 63.2 | ~34 |
| CK2β1 | 33.4 | 52.9 | ~72 |
| CK2β2 | 33.1 | 53.1 | ~71 |
| CK2β3 | 31.9 | 54.2 | ~65 |
| CK2β4 | 32.7 | 53.7 | ~68 |
| α1β1 Holoenzyme | 147.5 | 44.4 | ~127 |
| α1β2 Holoenzyme | 146.8 | 42.6 | ~141 |
| α1β3 Holoenzyme | 144.5 | 43.3 | ~135 |
| α1β4 Holoenzyme | 146 | 41.1 | ~154 |

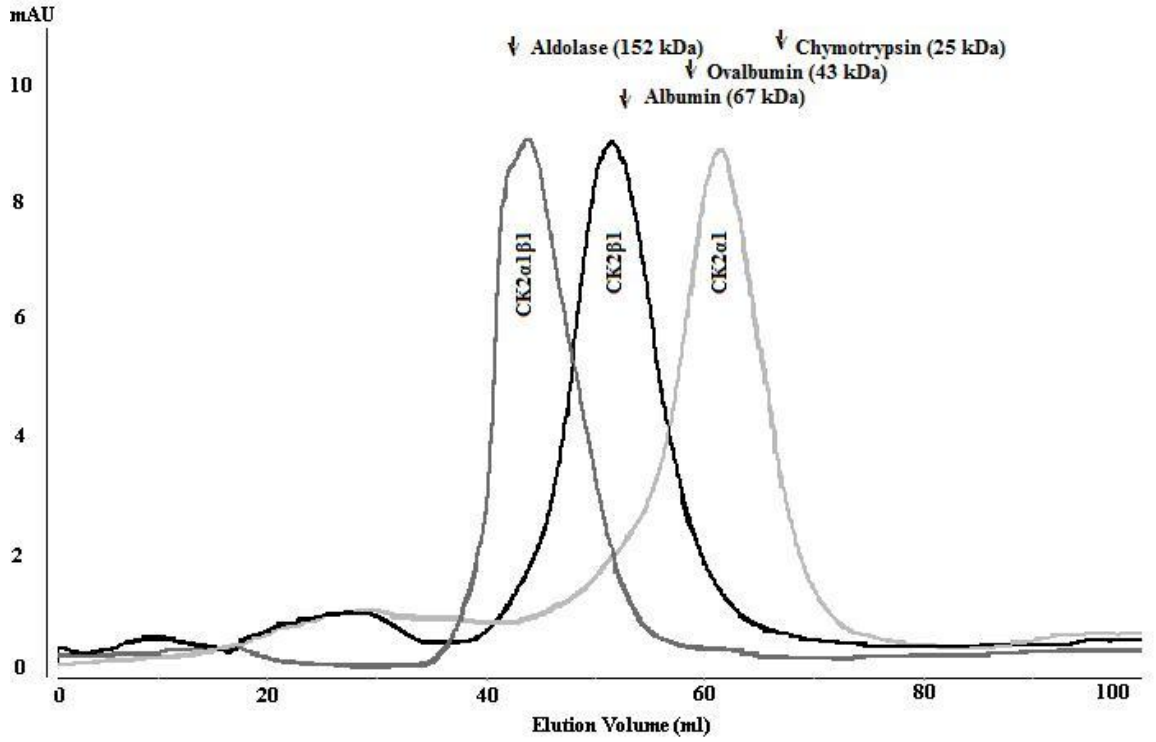



**Figure 2.3. Gel filtration analysis of CK2**

CK2α1, CK2β1 and CK2 holoenzyme show distinct molecular weight differences when analyzed by FPLC gel filtration using Sephacryl S-200 HR at 150 mM KCl. Protein elution from the 3 separate column runs was monitored by $A_{280}$. CK2α1 ( ; predicted MW 40.3 kDa) migrates as a monomer, while CK2β1 ( ; 33.4 kDa) migrates as a dimer. When combined CK2α1 and β1 elute as a single high molecular weight peak ( ), indicating formation of the holoenzyme product. Elution volumes for molecular size markers are indicated with arrows.

A native gradient gel was also run on catalytic CK2α1 and regulatory CK2β1 subunit alone and the eight CK2 holoenzyme tetramers (α1β1, α1β2, α1β3, α1β4, α2β1, α2β2, α2β3, α2β4). While it is difficult to detect a clear band for individual subunits alone, when combined in a 1:1 molar ratio, each holoenzyme migrates through the gel as a distinct high molecular weight band suggesting the formation of the holoenzyme

(Figure 2.4). Further support of holoenzyme formation is provided by β-subunit autophosphorylation. Previous studies have demonstrated that CK2 holoenzyme formation precedes β-subunit autophosphorylation (Pagano et al., 2005). All combinations of recombinant *A. thaliana* holoenzymes exhibit autophosphorylation of the CK2β subunit when incubated with either CK2α1 or CK2α2 (Figure 2.5). These results taken together show that all four isoforms of recombinant *A. thaliana* CK2β are capable of forming holoenzymes with either CK2α1 or CK2α2 *in vitro*.

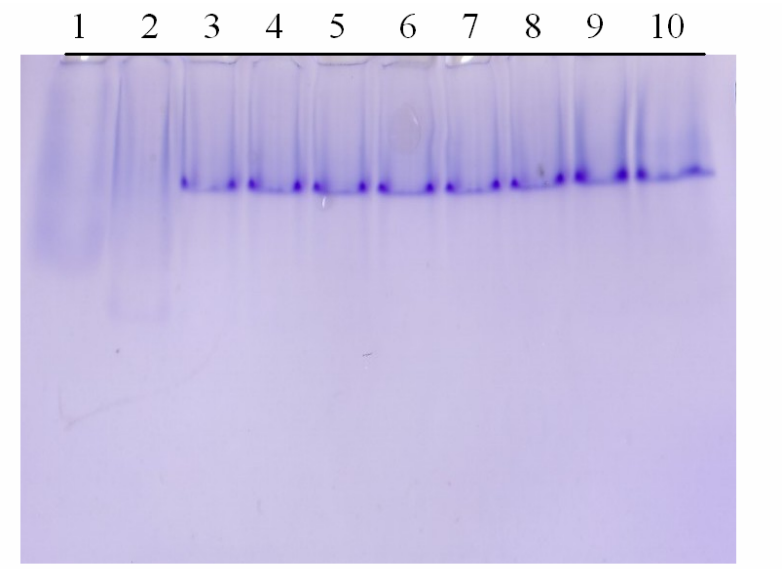



**Figure 2.4. Native gel electrophoresis of CK2 holoenzymes**

10 pmoles of CK2α1, CK2β1, and each CK2 holoenzyme tetramer were analyzed using non-denaturing gel electrophoresis. Upon formation of the holoenzyme, CK2 subunits migrate as a single high molecular weight band. Lane 1, CK2α1 alone; Lane 2, CK2β1 alone; Lane 3, CK2α1β1; Lane 4, CK2α1β2; Lane 5, CK2α1β3; Lane 6, CK2α1β4; Lane 7, CK2α2β1; Lane 8, CK2α2β2; Lane 9, CK2α2β3; Lane 10. CK2α2β4.

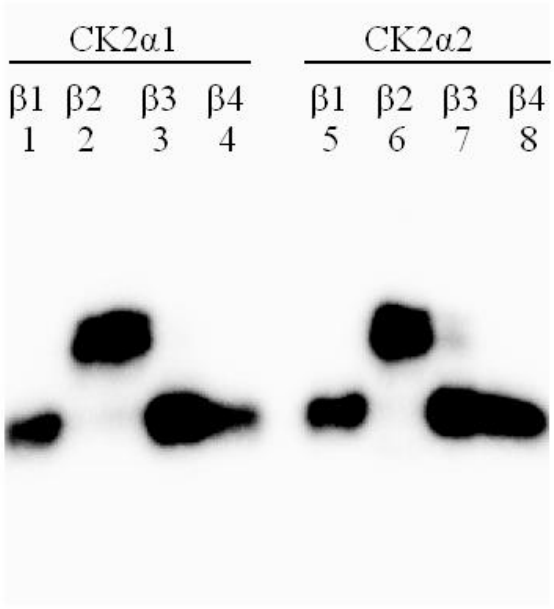



**Figure 2.5. Autophosphorylation of *A. thaliana* CK2β subunits**

Phosphorimage of CK2β subunit autophosphorylation following incubation with CK2α1 or CK2α2. Each 100 µl reaction contained 450 pmol of β-subunit (CK2β1, CK2β2, CK2β3, or CK2β4), 450 pmol of either CK2α1 or CK2α2, 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~250 cpm/pmol) and ~150 mM KCl. Each lane contains ~4 pmol of holoenzyme complex: Lane 1, CK2α1β1; Lane 2, CK2α1β2; Lane 3, CK2α2β3; Lane 4, CK2α1β4; Lane 5, CK2α2β1; Lane 6, CK2α2β2; Lane 7, CK2α2β3; Lane 8, CK2α2β4.

### 2.3.4. Catalytic Activity of Recombinant *A. thaliana* CK2 Subunits

Recombinant *A. thaliana* CK2α1 and CK2α2 exhibit catalytic activity similar to the native wheat germ CK2α (Figure 2.6). It was been previously demonstrated that wheat germ kinase, identified in this study as CK2α, phosphorylates an endogenous wheat germ protein termed T-substrate (Yan and Tao, 1982a). A homogenous purification of the 37 kDa wheat germ kinase was previously achieved using T-substrate to form an affinity matrix (Yan and Tao, 1982b). We identified T-substrate in this study as histone deacetylase 2β (HD2β). *A. thaliana* HD2β cDNA was cloned, expressed and purified to homogeneity (Figure 2.6). Both recombinant *A. thaliana* CK2 catalytic subunits and endogenous wheat germ CK2 phosphorylate native wheat HD2β (T-substrate) and recombinant *A. thaliana* HD2β to a similar extent *in vitro* (Figure 2.6).

HD2β is a member of the plant specific class of histone deacetylases (HD2 class) that have been shown to directly repress transcription by targeting to promoters *in planta* (Zhou et al., 2004). Histone deacetylases repress transcription by deacetylating the amino-terminal lysine residues on nucleosomal histones, thus facilitating the interaction of transcriptionally silent DNA with histones. Mammalian HDAC2 phosphorylation by CK2 promotes enzymatic activity and selectively regulates histone complex formation (Tsai and Seto, 2002). It has recently been suggested that CK2 phosphorylation of histone deacetylases (HDAC1 and HDAC2) may be the key mediator of aberrant HDAC activity observed in many types of cancer (Pluemsampant et al., 2008). While maize histone deacetylase HD2 has been shown to be an acidic nucleolar phosphoprotein (Lusser et al., 1997), this is the first study to demonstrate the phosphorylation of plant

histone deacetylases by CK2. Based on this finding it is tempting to speculate that plant histone deacetylases are also regulated post-translationally by CK2.

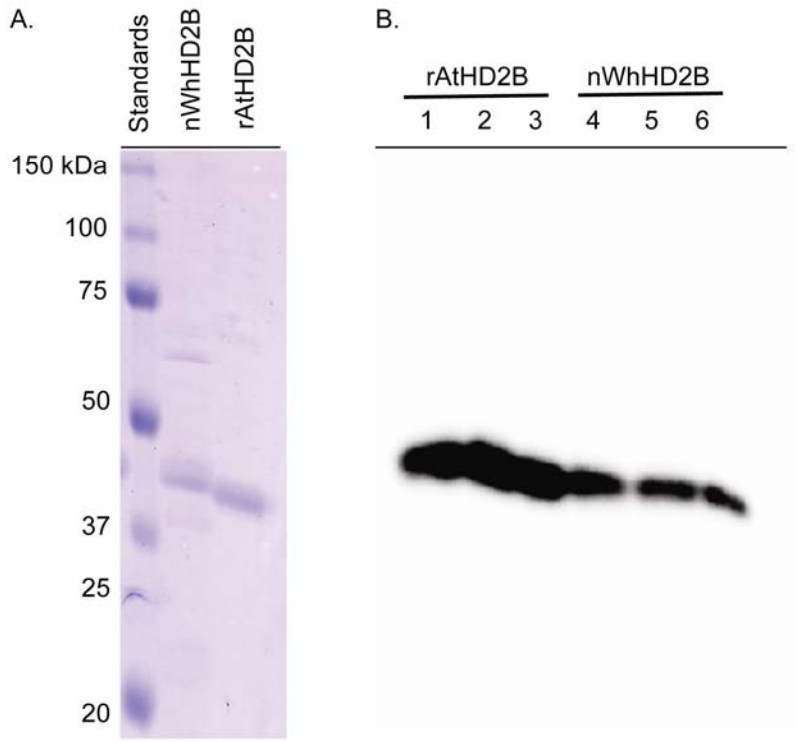



**Figure 2.6. Phosphorylation of HD2β by native and recombinant CK2**

A. SDS-PAGE analysis of HD2β. Lane 1. Precision protein standards (Bio-Rad) as indicated. Lane 2. 2 µg recombinant *A. thaliana* HD2β (rAtHD2β), Lane 3. 2 µg native wheat HD2β/T-substrate (nWhHD2β). B. Phosphorimage of rAtHD2β and nWhHD2β phosphorylation by 1 pmol recombinant *A. thaliana* CK2α1 (Lanes 1 and 4), recombinant *A. thaliana* CK2α2 (Lanes 2 and 5), or the native wheat germ CK2 preparation (Lanes 3 and 6). Each 20 µl reaction contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM[$\gamma$-$^{32}$P]ATP (~250 cpm/pmol), 100 mM KCl and 40 pmoles of HD2β. Reactions were incubated for 20 min at 37$^{o}$C and analyzed by SDS-PAGE.

### 2.3.5. Phosphorylation of Wheat Initiation Factors by CK2

The phosphorylation of various initiation factors by CK2 has been previously reported in yeast and mammalian systems; however, the extent of plant initiation factor phosphorylation by CK2 had not been established. A survey for additional CK2 substrates was conducted with various plant initiation factors. Recombinant *A. thaliana* CK2 subunits were unable to phosphorylate recombinant wheat eIF1, eIF1A, eIF4F, eIFiso4F or eIF4A; however, recombinant wheat eIF2α, eIF2β, eIF3c, eIF4B, and eIF5 were all phosphorylated by the kinase *in vitro* (Figure 2.7). While the phosphorylation of eIF2α and eIF3c had been previously demonstrated with the endogenous wheat germ kinase and native wheat eIF2 and eIF3, this is the first report that several other initiation factors serve as substrates of CK2 in plants, namely eIF2β, eIF4B, and eIF5. In addition, the phosphorylation of wheat novel cap binding protein (nCBP) by CK2 was also observed (data not shown). The phosphorylation of the native wheat eIF2β subunit by the endogenous wheat germ kinase (CK2α) was not previously observed, as phosphorylation of eIF2β by CK2 is negligible in the absence of regulatory CK2β subunits.

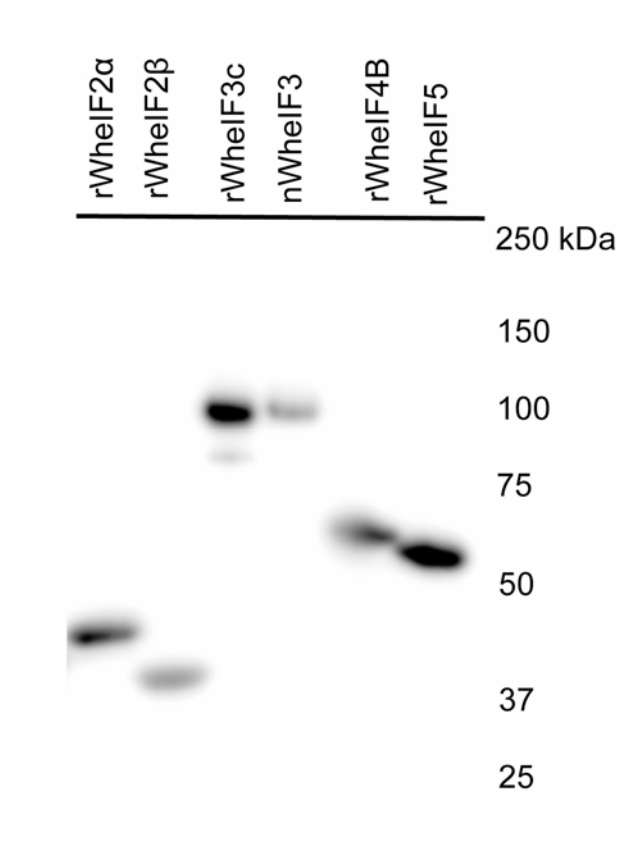



**Figure 2.7. Phosphorylation of eIF2, eIF3, eIF4B, and eIF5 by CK2**

Phosphorimage of wheat germ initiation factors phosphorylated by recombinant CK2. Each 20 µl reaction contained 50 mM Hepes-KOH, pH 7.6, 5 mM MgCl2, 2.4 mM DTT, 0.2 mM[γ-$^{32}$P]ATP (~250cpm/pmol), 100 mM KCl and the following wheat initiation factors: Lane 1, recombinant wheat eIF2α (50 pmol); Lane 2. recombinant wheat eIF2β (50 pmol); Lane 3, recombinant wheat eIF3c (25 pmol); Lane 4, native eIF3 (10 pmol); Lane 5. recombinant eIF4B (15 pmol); Lane 6. recombinant eIF5 (15 pmol). To optimize CK2 phosphorylation eIF2α, eIF3c, eIF3, eIF4B, and eIF5 were incubated in the presence of ~1 pmol of recombinant *A. thaliana* CK2α1; while eIF2β (Lane 2) was phosphorylated by ~0.25 pmol of the CK2α1β1 holoenzyme.

The presence of multiple subunits of CK2α and CK2β suggests that there might be differential activity of combinations of subunits or individual CK2α subunits. Each CK2 holoenzyme and individual CK2α subunit was tested for activity against the initiation factor substrates. Both recombinant *A. thaliana* CK2α subunits were catalytically active in the absence of CK2β toward HD2β, eIF3c, eIF4B, eIF5, and eIF2α. However, the phosphorylation of eIF2β by CK2α is negligible in the absence of CK2β. Upon holoenzyme formation, phosphorylation of eIF2β increases by 4 to 11-fold in the presence of various CK2β subunits (Figure 2.8). This increase is most dramatic in the presence of CK2β4. A similar increase in activity is seen with CK2α2; however, with these catalytic isoforms the increase in activity is more pronounced with CK2β1 (7-fold) as opposed to CK2β4 (2-fold). This may indicate a differential effect of the various CK2β subunits on the activity of the catalytic subunit isoforms toward individual substrates. In contrast, the presence of CK2β subunits reduces the phosphorylation of both native and recombinant wheat eIF3c by CK2α subunits (Figure 2.8). Native wheat eIF3 contains 13 non-identical subunits, and only eIF3c is phosphorylated by CK2. The reduction in eIF3c phosphorylation is most dramatic in the case of CK2α1 holoenzyme formation with CK2β1, which results in a 7-fold drop in activity. Similar results for eIF3c are seen with other CK2 subunits, as holoenzyme formation reduced activity of the catalytic subunits by 1.5 to 7-fold. Since eIF3c was estimated to contain approximately 7 phosphorylation sites, it is possible that CK2β subunits have specificity for certain sites. Interestingly, the phosphorylation of native wheat germ eIF3c, which is complexed with 12 other eIF3 subunits, shows a more dramatic reduction in phosphorylation in the

presence of CK2β subunit than recombinant eIF3c. This presents the possibility that other eIF3 subunits may prevent access to certain sites or sites are already phosphorylated in native eIF3. In the context of the multifactor complex, yeast eIF1 binds to eIF2β, eIF5, and eIF3c. Recombinant wheat eIF1 does not appear to be phosphorylated by CK2, however, the addition of excess eIF1 to phosphorylation assays reduces the phosphorylation of native eIF3 by CK2 subunits; however, no similar effect could be demonstrated on the phosphorylation of eIF5 or eIF2β (data not shown). It is suspected that the binding of eIF1 to eIF3c restricts access to CK2 phosphorylation sites.

The presence of CK2β produced a differential effect on eIF5 phosphorylation (Figure 2.8). The activity of CK2α1 and CK2α2 toward eIF5 was increased by the presence of CK2β3 (~30-50%); whereas the presence of CK2β1 reduced the activity of both catalytic subunits, although somewhat less so in the case of CK2α2 (~30% compared to ~20%). In addition the activity of CK2α1 toward eIF5 was reduced by complex formation with CK2β2 (~25%), and somewhat increased by CK2β4 (~20%). Meanwhile CK2β2 did not alter the phosphorylation by CK2α2, whereas complex formation with CK2β4 reduced its activity (~25%). No effect of holoenzyme formation on activity was detected for recombinant eIF2α or eIF4B phosphorylation (Figure 2.8). For both of these substrates CK2α subunits alone and the various holoenzymes showed no significant difference in phosphorylation. These results indicate that for some substrates the differential expression or availability of plant CK2 subunits may affect phosphorylation of substrates and thus provide differential regulation.

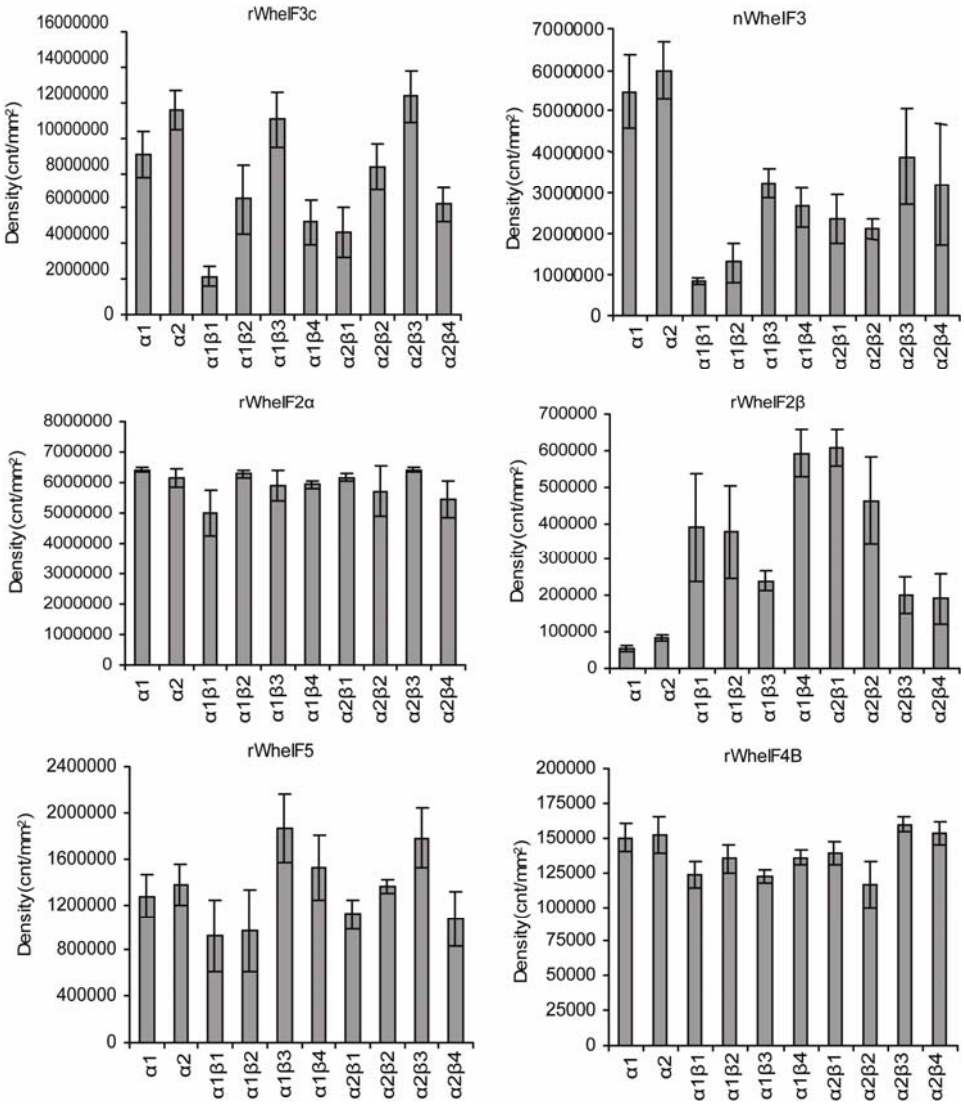



**Figure 2.8. Phosphorylation of wheat initiation factors by CK2 holoenzymes**

CK2 holoenzymes were incubated with various wheat initiation factors to compare activity. Each wheat initiation factor rWheIF3c (15 pmol), nWheIF3 (5 pmol), rWheIF2α (25 pmol), rWheIF2β (25 pmol), rWheIF5 (25 pmol), rWheIF4B (25 pmol) was incubated with 0.5 pmol of CK2α or 0.25 pmol of holoenzyme (tetramer containing 0.5 pmol CK2α complexed with 0.5 pmol CK2β) in 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM $\gamma[^{32}P]$-ATP (~250 cpm/pmol), and 100 mM KCl. at 25°C for 15 min. Samples were separated by SDS-PAGE, and analyzed by phosphorimaging to quantify incorporation of ATP into the substrate. Each reaction was performed in triplicate. Units are arbitrary and are expressed as counts/mm$^2$. Error bars represent +/- 1 standard deviation.

The approximate number of phosphorylation sites on each initiation factor was determined by the incorporation of [$^{32}$P] into each protein (Figure 2.9). CK2α1 was used to phosphorylate eIF2α, eIF3c, eIF4B, and eIF5, while CK2α1β4 was used to phosphorylate eIF2β. This decision was made based on the optimum combination of subunits for phosphorylation observed in Figure 2.8. At saturation, approximately 1 mole of phosphate is incorporated per mole of recombinant eIF2α and eIF2β (0.97 $\pm$ 0.04 and 0.84 $\pm$ 0.07, respectively). For eIF3c, eIF4B, and eIF5 quantitative analysis indicates multiple phosphorylation sites of 7.37 $\pm$ 0.58, 1.75 $\pm$ 0.07, and 3.1 $\pm$ 0.11 respectively. The presence of multiple sites suggests the phosphorylation of certain initiation factors may be regulated in a hierarchical manner. The identification of these sites is explored further in Chapter 3.

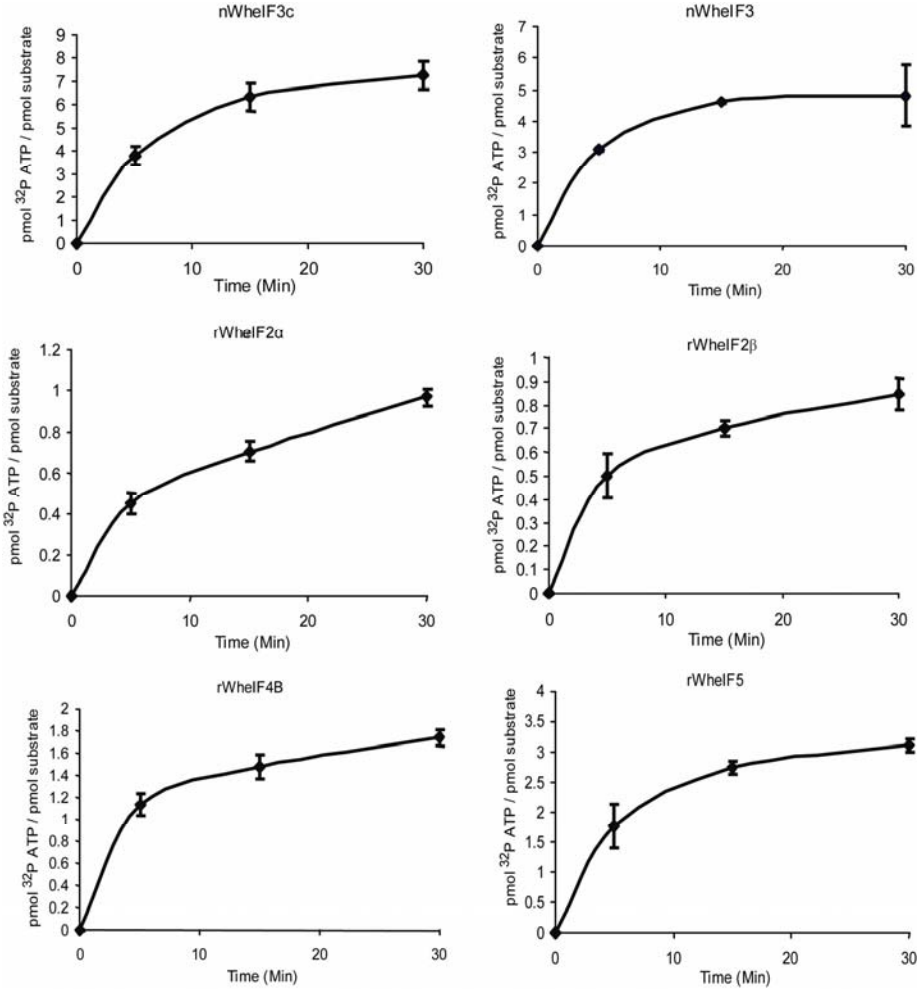



**Figure 2.9. Quantitative phosphorylation of wheat initiation factors by CK2**

The number of phosphorylation sites in each wheat initiation factor was estimated by the ability of CK2 to incorporate [γ-$^{32}$P]-ATP into each substrate. rWheIF3c (150 pmol), nWheIF3 (50 pmol), rWheIF2α (250 pmol), rWheIF2β (250 pmol), rWheIF5 (250 pmol), rWheIF4B (250 pmol) was incubated with kinase in 50 µl reactions containing ~5 pmol of recombinant CK2α1, 5 µg of acetylated BSA, 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [γ-$^{32}$P]ATP (~100 cpm/pmol), and 100 mM KCl for 10, 20, or 30 min at 30°C. Reactions were stopped by the addition of HMK, samples were immediately captured on a nitrocellulose filter, and quantified using a scintillation counter. Each reaction was run in triplicate and the results averaged. Error bars represent +/- 1 standard deviation. For rWheIF3c, nWheIF3, rWheIF2α, rWheIF5, rWheIF4B CK2α1 was used to phosphorylate the substrate. The efficient phosphorylation of rWheIF2β requires CK2 holoenzyme, and the complex of CK2α1β1 was used for its phosphorylation. Since CK2β1 undergoes autophosphorylation, a separate blank was run for this reaction.

## 2.4. CONCLUSIONS

We have successfully expressed and purified 6 CK2 subunits from *A. thaliana* and shown that they have enzymatic activity similar to the wheat native CK2. In addition to phosphorylating eIF2α and eIF3c, CK2 was observed to phosphorylate eIF2β, eIF4B, eIF5, nCBP, and histone deacetylase 2β (HD2β). CK2 was not found to phosphorylate wheat eIF1, eIF1A, eIF4A, eIF4F, or eIFiso4F. In previous studies, the phosphorylation of native wheat eIF2β was not observed in the presence of native wheat germ kinase, which consisted of only the catalytic CK2α subunit. Similar findings were observed with recombinant *A. thaliana* CK2 subunits, where phosphorylation of eIF2β only occurs in the presence of regulatory CK2β subunits. The work presented demonstrates that holoenzyme formation of catalytic CK2α subunits with various regulatory CK2β subunits alters the ability of *A. thaliana* CK2 to phosphorylate certain plant initiation factors *in vitro*. More specifically, when assembled into holoenzymes with CK2β, CK2α subunits exhibit an increase in the phosphorylation of eIF2β; however, a reduction in the ability to phosphorylate eIF3c was observed suggesting differential regulation by the availability of various CK2 subunits. The magnitude of these alterations in catalytic activity is not equivalent for all regulatory subunits. This clearly presents a potential mechanism by which the expression of CK2β subunit isoforms could alter substrate phosphorylation.

Since CK2 is typically viewed as constitutively active, the regulation of CK2 β-subunit expression in plants presents a novel mechanism for regulating kinase activity. It has been previously shown that *A. thaliana* CK2β subunits exhibit differential subcellular distribution (Salinas et al., 2006), and DNA array data indicates that *A. thaliana*

regulatory CK2β subunit mRNAs are differentially expressed in specific tissues during development and in response to stresses (http://www.bar.utoronto.ca/). This supports similar findings in maize that demonstrate the differential expression of three maize CK2β isoforms during development (Riera et al., 2001b) and the plant cell cycle (Espunya et al., 2005). The phosphorylation data presented here supports the possibility that expression of CK2β subunits in plants could be used to modify the substrate specificity of the constitutively expressed CK2α catalytic subunits toward various substrates *in vivo*.

It has been established that a stable multifactor complex (MFC) consisting of eIF3/eIF1/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$ forms independent of ribosomal subunits and is an important translation initiation intermediate in yeast (Asano et al., 2000). Interestingly, CK2 has been shown to phosphorylate four key subunits involved in the formation of this complex: eIF3c (Browning et al., 1985), eIF5 (Majumdar et al., 2002), eIF2α (Feng et al., 1994), and eIF2β (Llorens et al., 2005b). This is the first study to show that all four subunits from a single system are phosphorylated by CK2. In yeast, the N-terminal domain of eIF3c/NIP1 has been shown to interact directly with both eIF1 and eIF5 (Valasek et al., 2004). A mass spectroscopic analysis of human eIF3 post-translational modifications revealed 8 phosphorylation sites in the NTD of eIF3 with signature CK2 phosphorylation domains (Damoc et al., 2006). Wheat eIF3c contains an astonishing 15 potential CK2 phosphorylation sites (http://scansite.mit.edu/) all clustered in the N-terminal domain (NTD), and quantitation of CK2 phosphorylation (see Figure 2.9) suggests that at least 7 of these potential sites are phosphorylated simultaneously *in vitro*. Since positively charged domains in yeast eIF1 and eIF5 are believed to be

responsible for binding to eIF3c (Yamamoto et al., 2005; Reibarkh et al., 2008), it is suspected that phosphorylation of the eIF3c N-terminal domain could alter the ability to bind to eIF1 and eIF5. By regulating the phosphorylation of eIF3c, it is possible that CK2 holoenzyme formation influences multifactor complex formation and could thus influence protein translation. Furthermore, there is evidence that CK2 phosphorylation of mammalian eIF5 plays a critical role in the multifactor complex formation by facilitating the interaction of eIF5 with eIF2. CK2 phosphorylates two serine residues in an Aromatic/Acidic box-2 (AA-box) on the C-terminus of mammalian eIF5 in response to cell cycle progression (Homma and Homma, 2005). It has been demonstrated that the binding of mammalian eIF5 to eIF2 occurs through electrostatic interactions between the K-boxes of eIF2β and the AA-boxes of eIF5's HEAT domain, and phosphorylation of eIF5 by CK2 is believed to facilitate this interaction (Yamamoto et al., 2005). Mutations that prevent the phosphorylation of these residues inhibit the formation of mature multifactor complexes (MFC) consisting of eIF1, eIF2, eIF3, and eIF5 and disturb synchronous progression of cells through S to M phase (Homma et al., 2005). Critical binding domains involved in multifactor complex formation are conserved in plants, and similar interactions are likely to occur. In plants the regulation of these interactions by phosphorylation takes on the added complexity of multiple CK2 isoforms that are differentially expressed. These findings taken together suggest a potential role for CK2 isoforms in regulating translation initiation.

# Chapter 3: Identification of CK2 Phosphorylation Sites in Plant Initiation Factors

## 3.1. INTRODUCTION

Protein kinase CK2 (formerly known as casein kinase II) is a ubiquitous and highly conserved serine/threonine kinase found in both the nucleus and cytoplasm of all eukaryotic cells (Pinna, 1997; Litchfield, 2003; Olsten and Litchfield, 2004; Pinna, 2006). CK2 is essential for cell viability, and is involved in key processes such as transcriptional and translational control, cell proliferation, cell cycle progression, and apoptosis (Litchfield, 2003). Unlike the majority of protein kinases, whose activity is dependant on some form of stimulus or effector, the activity of CK2 is independent of second messengers, phosphorylation events, or even holoenzyme formation (Pinna, 2006). It is the goal of this study to identify the residues phosphorylated by CK2 in various plant translation initiation factors.

The activities of the initiation factors involved in translation initiation are highly regulated through the phosphorylation of the translational machinery (Hershey, 1989; Proud, 1992; Gingras et al., 2004; Proud, 2007). The phosphorylation of various factors leads to both modulations in their activities and alterations in their interactions with one another and other components of the cellular machinery (Proud, 2007). However, the phosphorylation of initiation factors has not yet been well characterized in plants or other organisms. Wheat germ extracts contain an active form of CK2 capable of phosphorylating a number of the factors (eIF2α, eIF2β, eIF3c, eIF4B, nCBP, and eIF5;

see Chapter 2) and some evidence exists that phosphorylation of these factors by CK2 in mammalian cells may regulate cell cycle progression (Homma et al., 2005).

A number of CK2 phosphorylation sites have been identified in initiation factors from various systems, namely in yeast and mammalian cells; however, it is not known if similar phosphorylation sites exist in plants (Meggio and Pinna, 2003). Variations in the phosphorylation of substrates between these systems have been known for some time. In yeast, the N-terminal domains of mRNA cap-binding proteins eIF4E and p20 are phosphorylated by CK2; however, no similar sites are seen in mammalian or plant eIF4Es (Zanchin and McCarthy, 1995), nor do their cap-binding proteins appear to be phosphorylated by CK2 *in vitro* (Chapter 2). Interestingly, plant nCBP does appear to be phosphorylated by CK2 *in vitro* (unpublished observation; MDDennis). In addition, some variations are known to exist in the phosphorylation of eIF2 by CK2. In mammalian cells, only the β-subunit of eIF2 is phosphorylated by CK2 *in vivo* (Llorens et al., 2005a). The primary phosphorylation site in mammalian eIF2β (S2) is not conserved in either plants or yeast, yet CK2 still phosphorylates other residues within eIF2 complexes in these systems. In yeast, only phosphorylation of the eIF2α subunit has been reported *in vivo* (Feng et al., 1994). Meanwhile, both the α and β subunits of wheat eIF2 were reported to be phosphorylated by sea star CK2 holoenzyme *in vitro* (Janaki et al., 1995). In this study, recombinant wheat translation initiation factors were used to identify *in vitro* CK2 phosphorylation sites in eIF2, eIF3, eIF4B, and eIF5. In addition, the phosphorylation status of an endogenous wheat germ eIF5 preparation was also evaluated to provide evidence of *in vivo* factor phosphorylation.

## 3.2. METHODS

### 3.2.1. Materials

All chemicals were of high quality and obtained from Sigma, Fisher, VWR or as indicated. Ni-NTA Superflow (Qiagen) was used according to manufacturer's instructions to purify 6XHis tagged substrates. The buffers used for chromatography were Buffer B (20 mM HEPES-KOH pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and KCl as indicated, Buffer B-100 is Buffer B containing 100 mM KCl), Buffer C (50 mM HEPES-KOH pH 7.6, 600 mM KCl, 20 mM imidazole) and Buffer D (20 mM HEPES-KOH pH 7.6, 250 mM imidazole, and KCl as indicated).

### 3.2.2. Cloning, Expression, and Purification of Recombinant Translation Initiation Factors and CK2

To obtain non-phosphorylated plant initiation factors for analysis, wheat initiation factors that were identified as CK2 targets were cloned and expressed in *E. coli*. The cloning and expression of wheat eIF2α, eIF2β, eIF3c, eIF4B, and eIF5 were previously described (Metz and Browning, 1997; Mayberry et al., 2007) and see Chapter 2).

To obtain the N-terminal domain of eIF3c (eIF3c-NTD), the coding region from the wheat-eIF3c-pET23d(+) construct (Chapter 2) was cleaved with *NcoI/NdeI* to produce a 947 bp fragment that coded for the N-terminal 318 amino acids of eIF3c. This fragment was cloned into pET15b(+) using *NcoI/NdeI* and then re-cut using *NcoI/XhoI*. The new *NcoI/XhoI* eIF3c N-terminally truncated fragment was cloned into a new pET23d(+) vector, such that a C-terminal 6xHis tag and stop codon were added to the coding sequence. The construct was confirmed by sequencing and transformed into

BL21(DE3) *E. coli* cells. A 50 ml culture of LB was inoculated with a single colony and grown overnight; 25 ml of culture was then used to inoculate 2 X 1L LB, which was grown at 37°C to an $A_{600}$ of 0.5. The temperature was then reduced to 30°C and 0.5 mM IPTG (isopropyl β-D-thiogalactoside) was added to induce expression. Protein expression was allowed to continue for 3 hr at 30°C, and cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. The cells were resuspended in 20 ml of Buffer C containing 1 small Complete protease mini inhibitor tablet (Roche) and disrupted by sonication for 3 X 30 sec at 70% power and 2 x 90% power using a Vibra Cell sonicator (Sonics & Materials Inc.). Lysed cells were centrifuged at 184,048 *g* for 30 min at 4°C. The supernatant was filtered through a 0.2 micron filter (Millipore) and loaded onto a 2 ml column of Ni-NTA (Qiagen). The column was washed with 20 ml of Buffer C, and the recombinant eIF3c-NTD was eluted with Buffer D. The elution was collected in 1 ml fractions and 10 µl of each fraction was analyzed by SDS-PAGE to evaluate the purity of the recombinant eIF3c-NTD. The cleanest fractions (6 ml) were pooled and dialyzed overnight at 4°C against Buffer B-100.

The cloning and expression of *A. thaliana* CK2 subunits was as described (Chapter 2).

### 3.2.3. Site-Directed Mutagenesis of eIF2α and eIF5

To confirm the phosphorylation sites identified by mass spectrometry, site-directed mutagenesis was performed on eIF2α and eIF5. For each CK2 phosphorylation site a forward and reverse primer was designed to alter the phosphorylated serine

/threonine to alanine. In each case a 50 µl PCR reaction contained the following: 2.5 U FastStart High Fidelity Enzyme Blend (Roche), 5 µl FastStart Reaction Buffer, 200 µM dNTP mix, 10 pmol of each primer, and plasmid template. A series of 3 reactions were performed to obtain each mutated residue using the following primer sets: Reaction 1 contained the 5′ forward coding region primer and the 3′ reverse mutated residue primer; Reaction 2 contained the 5′mutated residue primer and the 3′ reverse coding region primer; Reaction 3 contained DNA products from Reaction 1 and Reaction 2. For a list of primers used see Appendix Table 3.2. PCR products with the desired mutations were then cloned back into pET15b using *NcoI/BamHI*. This process was used to make clones containing point mutations at all three CK2 phosphorylation sites identified in wheat eIF5 (S209, T240, & S452) and at S318 in wheat eIF2α. To make eIF5 with multiple mutations, digests were performed on single residue mutants using *NdeI/BsrGI* and *BmgBI/BamHI*, and the segments containing point mutations were cloned together. This process produced the triple alanine mutant (eIF5AAA) in which all 3 CK2 phosphorylation sites have been removed. All mutant forms of His-eIF5 were expressed and purified as previously described for the wild-type His-eIF5 using Ni-NTA and phosphocellulose (Mayberry et al., 2007). eIF2α and eIF5 containing the desired mutations were expressed in BL21(DE3) *E. coli* and purified as previously described for the wild-type forms of the proteins (Chang et al., 1999; Mayberry et al., 2007).

### 3.2.4. Purification of Native Wheat eIF5

Native eIF5 was purified from wheat germ using methods similar to those previously described (Lax et al., 1986). Wheat germ (200 g) was ground in a cold blender and mixed with 200 ml of extraction buffer (50 mM Hepes-KOH pH 7.6, 120 mM KCl, 2 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 6 mM BME, 0.65 mM STI, 0.5 mM PMSF, 20 nM calyculin A (BioSource) and centrifuged for 20 min at 15,000 x *g* at 4° C. Calyculin A is a marine toxin that specifically inhibits protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) activity (Ishihara et al., 1989). Following centrifugation, the fatty layer was removed by pouring the sample through cheesecloth and spun again for 3 hrs at 218,222 x *g* at 4° C. The supernatant (~150 ml) was then saturated to 40% with ammonium sulfate, and the precipitated proteins were removed by centrifugation at x g for 10 min at 4° C. A second ammonium sulfate fraction was obtained from 40-70-% saturation, and the proteins were again collected by centrifugation. The proteins that precipitated in the 40-70% fraction were resuspended in approximately 25 ml of Buffer B-50 and 20 nM calyculin A. Dialysis was performed for 8 hours with 3 changes of 500 mL Buffer B plus 50 mM KCl and 20 nM calyculin A. The supernatant was then applied an 20 ml Fast Flow DEAE-Sepharose column equilibrated in Buffer B-50 and 20 nM calyculin A. The column was washed with Buffer B-50 and 20 nM calyculin A until the $A_{280}$ returned to baseline and eIF5 was eluted from the column using a 200 ml gradient from 100 mM to 800 mM KCl in Buffer B plus 20 nM calyculin A. Fractions of 10 ml were collected, and 50 ul aliquots were taken of each fraction for analysis. Each fraction was analyzed by ELISA using rabbit antibodies raised to recombinant wheat eIF5

(Rαwt5; Sigma) and fractions containing the eIF5 peak were pooled. The peak eIF5 fractions (approximately 20 ml) were loaded onto a 2 ml phosphocellulose column equilibrated in Buffer B-150 plus 20 nM calyculin A. The column was washed with 20 ml of Buffer B-150 plus 20 nM calyculin A, and eIF5 was eluted with a 10X (20 ml) gradient from 150 mM KCl to 600 mM KCl. Fractions of 1 ml were collected and 10 µl of each fraction was analyzed by SDS-PAGE. The peak fractions containing eIF5 were pooled and concentrated to approximately 1 ml using an Amicon Ultra-4 centrifugal filter (Millipore).

### 3.2.5. Phosphorylation Site Identification

For *in vitro* phosphorylation, 2 µg of recombinant wheat substrate (eIF2α, eIF2β, eIF3c, eIF3c-NTD, eIF4B, and eIF5) was incubated in a 20 µl reactions with and without 50 pmoles of CK2 for 1 hour at 30 $^{o}$C. To optimize phosphorylation recombinant eIF2α, eIF3c, eIF4Br, and eIF5 were all phosphorylated by recombinant *A. thaliana* CK2α1. No phosphorylation of eIF2β is detected in the absence of the CK2β subunit, thus CK2α1β4 complex was used for phosphorylation of eIF2β (see Figure 2.8 for optimal CK2 forms). Each reaction contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM ATP, and 100 mM KCl. The reactions were loaded onto a 12.5% Bis-Tris gel, run using MOPS running buffer (Invitrogen) and stained with colloidal blue (Invitrogen). The substrate bands from both the kinase treatment and the no kinase reactions were cut from the gel, and prepared for analysis by mass spectrometry as previously described in

Chapter 2. All steps in the in-gel digest process were conducted in a laminar flow hood to prevent keratin contamination.

For *in vivo* site identification, the endogenous wheat germ eIF5 preparation was loaded directly onto a 12.5% Bis-Tris gel and the doublet representing the two isoforms of wheat eIF5 were treated using the above protocol for in gel trypsin digestion.

Phosphorylation site identification was performed at the University of Texas Institute for Cell and Molecular Biology Analytical Instrumentation Facility Core (Maria Person, Director) using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems). The digested protein samples were enriched for phosphopeptides using an immobilized metal affinity chromatography (IMAC) resin (Phos-Select, Sigma). The samples were treated according to the manufacturer's protocol, with some modifications. Both flow through and eluant (enriched) samples were analyzed by MALDI-MS, as well as the unseparated digest. Prior to MS analysis, each tryptic digest was dried to <5 μl in a SpeedVac (ThermoSavant), desalted with a Ziptip μ-C18 pipette tip (Millipore), and eluted with 1.5 μl of matrix onto three spots on the MALDI target. The matrix was 0.7 mg/ml α-cyano-4-hydroxycinnamic acid in 67% acetonitrile/0.2% trifluoroacetic acid. Calibration Mixture 4700 (Applied Biosciences) was prepared according to manufacturer's instructions.

MS spectra were obtained using 2000 laser shots over a mass range of 700-4000 and calibrated internally on trypsin autolysis peaks. MS spectra were acquired in linear and reflectron modes. The Phos-Select flow through, eluant, and unseparated MS were analyzed in both linear and reflectron modes. Phosphopeptides were indicated by a

decrease in signal in the reflectron mode, or the appearance of a neutral loss ion of poor resolution. Additionally, peptides enriched in the IMAC fraction were tested for the presence of phosphorylation by acquisition of MS/MS, where neutral loss of phosphoric acid is seen as the dominant fragment ion. MS spectra were also analyzed using MASCOT in the GPS Explorer software and peptides with masses corresponding to eIF phosphopeptides were subjected to MS/MS analysis. MS/MS fragmentation spectra were acquired for all phosphopeptides in order to determine the site of modification. Fragment ions with +80 Da mass shift indicated addition of a phosphate, and neutral loss of -98 Da fragment ions were also observed. For most peptides, the site(s) of phosphorylation were unambiguously assigned, but in some cases, a stretch of residues contained potential sites and no fragment ions were available to distinguish the exact site. When possible, the MS/MS of the corresponding unphosphorylated peptide was acquired and compared to the phosphopeptide as an additional tool to map the site of phosphorylation.

### 3.2.6. Phosphorylation of Initiation Factors *in vitro*

To verify phosphorylation sites using recombinant factors containing desired mutations, phosphorylation was conducted in 20 µl of a reaction mixture containing 5 µg of substrate, 0.5 µg of recombinant CK2α, and 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~200 cpm/pmol), and 100 mM KCl. Reactions were incubated at 30°C for 30 min, and terminated by the addition of 4x SDS loading dye (2.5 M Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 0.1% bromophenol blue). Proteins were separated by SDS-PAGE, and gels exposed to a phosphorimager screen (Kodak) for 12

hrs. The screen was then analyzed on a Molecular Imager FX using Quantity One software (BioRad).

## 3.3. RESULTS AND DISCUSSION

### 3.3.1. Identification of CK2 Phosphorylation Sites

For quite some time it has been known that wheat germ eIF2 and eIF3 were phosphorylated by an endogenous wheat germ kinase with casein kinase-like properties (Browning et al., 1985); however, the identity of this kinase and the initiation factor residues targeted for phosphorylation were unknown. The endogenous kinase responsible for the phosphorylation of wheat eIF2 and eIF3 was identified in this work as CK2 (Chapter 2). Further investigation into the phosphorylation of recombinant wheat initiation factors revealed that CK2 substrates were not only restricted to the eIF2α and eIF3c subunits, but also included wheat eIF2β, eIF4B, and eIF5. This is the first study to show that four subunits of the factors involved in forming the multifactor complex were all phosphorylated by CK2. In order to evaluate the potential effect CK2 phosphorylation has on translation initiation factors, it was necessary to identify the residues that were modified by the kinase. This is the first study to identify CK2 phosphorylation sites in all of these subunits. Our analysis of initiation factor phosphopeptides produced by *in vitro* phosphorylation has identified 20 CK2 phosphorylation sites in eIF2α, eIF2β, eIF3c, eIF4B, and eIF5 (Table 3.1), and it appears that at least some of these sites are phosphorylated in preparations of native wheat germ factors. Native wheat germ eIF5 purified in the presence of the phosphatase inhibitor calyculin A is multiply

phosphorylated. Two distinct isoforms of native eIF5 were identified and both were phosphorylated on the C-terminus at the residue corresponding to S451 (S449). Recombinant wheat eIF5 is phosphorylated on this residue by CK2 *in vitro*.

**Table 3.1. *In vitro* CK2 phosphorylation sites identified in wheat initiation factors**

| Substrate | CK2 Phosphorylation Sites |
|---|---|
| eIF2α | S318 |
| eIF2β | T52/S54, T85 |
| eIF3c | S11, S13, S22, S26, S28, S31, S53, S56, S198, S210, S212, S215 |
| eIF4B | S140, S241 |
| eIF5 | S209, T240, S451 |

**eIF2α**. Wheat eIF2α has 3 predicted CK2 consensus sites (www.scansite.mit.edu, scores of 0.51, 0.50, 0.18); however, only S318 is of high stringency. S318 was identified by mass spectrometry as the only CK2 phosphorylation site. This was then confirmed by site-directed mutagenesis of S318. Wild-type recombinant eIF2α is phosphorylated by CK2 (Figure 3.1, lane 1), however CK2 is no longer able to phosphorylate eIF2α S318A (lane 2).

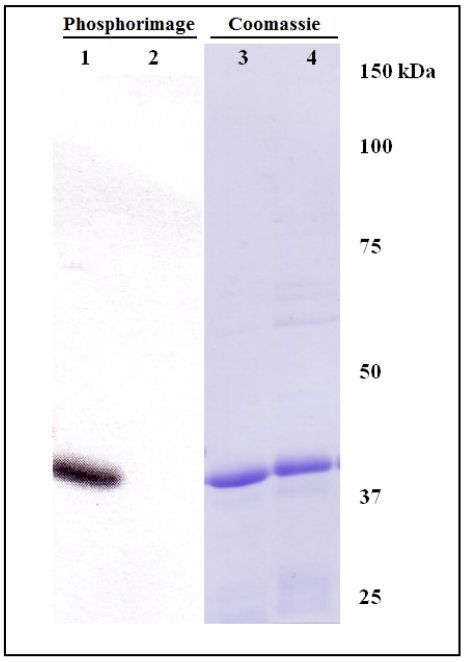



**Figure 3.1. Phosphorylation of eIF2α and eIF2α S318A by CK2**

Recombinant Wheat eIF2α and eIF2α S318A were incubated in the presence of CK2. Each 20 µl reaction contained 100 pmoles of eIF2α or eIF2α S318A, 5 pmoles of recombinant CK2α, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [γ-$^{32}$P]ATP (~200 cpm/pmol), and 100 mM KCl. Reactions were separated by SDS-PAGE, stained with Coomassie Blue and analyzed by phosphorimaging. Following incubation the recombinant factors were run on 12.5% SDS-PAGE and exposed to phosphorimager screen. Lanes 1 & 2 are from phosphorimager; lanes 3 & 4 are Coomassie stained.

**eIF2β.** Wheat eIF2β contains 3 consensus CK2 phosphorylation sites (www.scansite.mit.edu; T52, T85, T112, with scores of 0.59, 0.46, & 0.52, respectively). The mass of peptide *46*TEGLSV**T**E**S**GEASFVGLK*63* is shifted by a single phosphate; unfortunately, the MS/MS spectra for this region could not differentiate between T52 and S54. While only T52 is a consensus CK2 site, it cannot be ruled out that S54 is phosphorylated by CK2 *in vitro*. In addition to this site, T85 was also observed partially

phosphorylated. The peptide containing T112 was only detected in the non-phosphorylated form, and thus this residue does not appear to be phosphorylated by CK2 *in vitro*. Phosphorylation of plant eIF2β by CK2 is dependant on formation of the CK2 holoenzyme complex, as very little phosphorylation occurs in the absence of the regulatory subunit (see Chapter 2). Quantitative analysis of eIF2β phosphorylation suggests that less than 1 mol of phosphate ($0.87 \pm 0.07$) is incorporated into each mole of recombinant wheat eIF2β *in vitro* (see Chapter 2); surprisingly however, two unique phosphorylation sites were identified by mass spectrometry (T52/S54 and T85). Taken together, this suggests that the recombinant eIF2β is not homogeneously phosphorylated by the kinase. It is important to note that eIF2β is typically in the context of the eIF2 trimer (complexed with α and γ subunits) and some portion of the recombinant enzyme, while soluble, may not be properly folded in the absence of its other subunits. Improper folding could result in an apparent reduction in CK2 phosphorylation. It is also possible that the 2 CK2 sites in eIF2β may not be phosphorylated simultaneously.

**eIF3c.** eIF3c has 15 predicted CK2 consensus sites (www.scansite.mit.edu, score below 0.59). Initial attempts to identify the phosphorylation sites using full-length recombinant wheat eIF3c failed due to poor spectral coverage in the N-terminus. The N-terminal region of eIF3c is not only highly acidic, but it also contains a large number of phosphorylation sites and few tryptic cleavage sites. The large negative charge imparted by this combination makes the peptides from this region difficult to analyze using mass spectrometry. Two critical peptides, amino acids 5-38 & 45-62 could be clearly observed in the non-phosphorylated sample, but were undetectable in phosphorylated samples of full-length eIF3c. Even in negative ion mode, no phosphorylated version of the peptide

was seen. This is likely due to the formation of adducts with Na+ and Fe++ in the presence of multiple phosphorylation events. To overcome this problem and enrich for peptides specifically in this region, a 315-amino acid N-terminal truncation mutant of eIF3c was created (eIF3c-NTD). We identified 12 *in vitro* CK2 phosphorylation sites using recombinant CK2 to phosphorylate eIF3c-NTD, followed by IMAC enrichment for phosphopeptides and mass spectrometric analysis.

The MS spectra for eIF3c-NTD phosphorylation was also complicated by the presence of multiply phosphorylated states in combination with various Na+ and Fe++ adducts, which resulted in the appearance of peptide-adduct peak clusters with varying amounts of phosphorylation (Figure 3.2). The strongest peak formed by the phosphorylated peptide *5*FWGQGD**S**D**S**EEEVDEIE**S**EQG**S**D**S**EK**S**EAGDGGR*38* occurs at 3889 Da, suggesting 3 phosphorylation sites; however there are peaks for this cluster that correspond with the addition of up to 6 phosphates and $Fe^{++}$, suggesting that all six serines within this region (S11, S13, S22, S26, S28, and S31) can be phosphorylated by CK2 *in vitro* (Figure 3.2). The phosphorylation of S31 appears to inhibit trypsin cleavage at K30. Two phosphates also appeared to be incorporated into the peptide *49*YTQD**S**DD**S**DTESHR*62*, and based on the MS/MS for this peptide S53 and S56 were identified as *in vitro* CK2 phosphorylation sites. The mass of the eIF3c-NTD peptide from 174-207 was found to shift in the phosphorylated sample due to the presence of a single phosphorylation site, which was identified at S198. Similarly, peptide *208*MA**S**D**S**ED**S**GNEDDVSQDGGAWEK*230* was found to contain three phosphorylation sites, which were identified in the MS/MS as S210, S212, and S215.

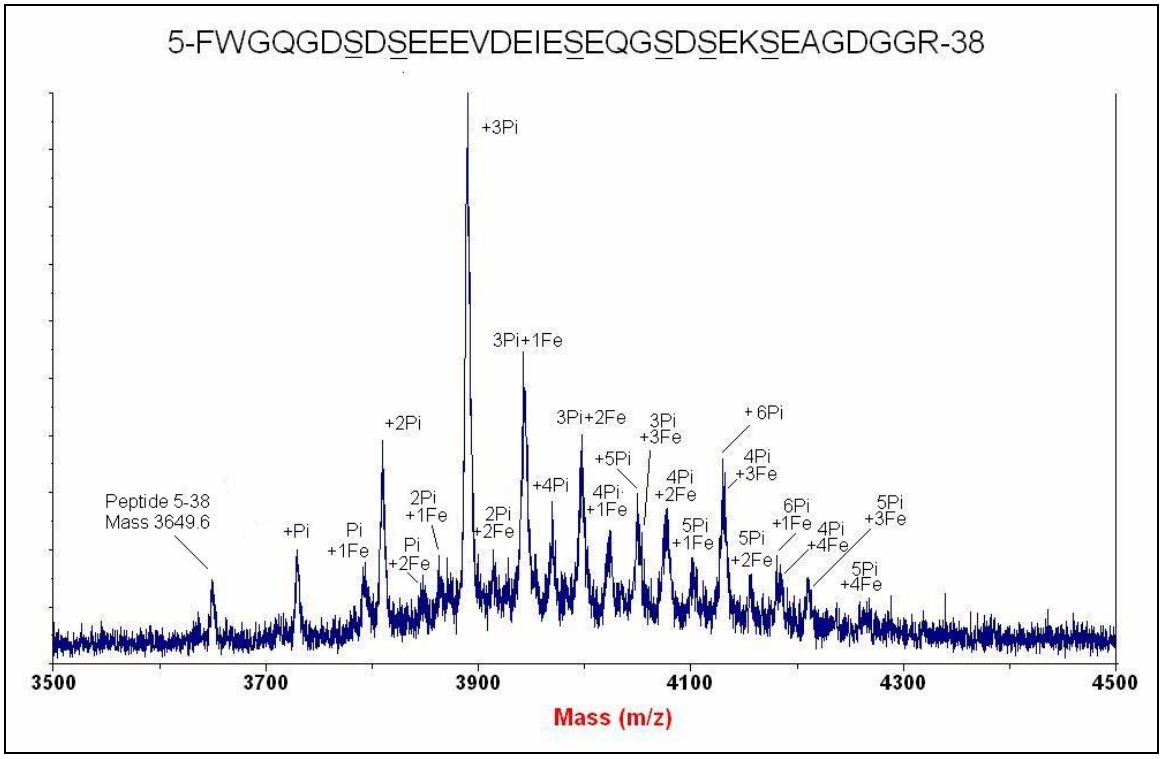



**Figure 3.2. Wheat eIF3c-NTD MS following *in vitro* CK2 phosphorylation**

MS analysis of eIF3c-NTD following *in vitro* CK2 phosphorylation. This peptide corresponds to *5*FWGQGDSDSEEEVDEIESEQGSDSEKSEAGDGGR*38,* which has an expected mass of 3649.6. The addition of phosphate shifts this mass by +80 Da, while the addition of $Fe^{++}$ is observed as +53 Da. The analysis of this sample was complicated by the formation of $Fe^{++}$ adducts, which resulted in the appearance of peptide-adduct peak clusters with varying amounts of phosphorylation. These adducts are believed to be caused by the extremely negative charge of this peptide following CK2 treatment. This spectra suggests that all six serines in this region (S11, S13, S22, S26, S28, and S31) can potentially be phosphorylated by CK2 *in vitro*. Calculated mass shifts are included in Appendix Table 3.3.

**eIF5**. Wheat eIF5 has 5 predicted CK2 consensus sites (www.scansite.mit.edu scores below 0.59). We have unambiguously identified three distinct CK2 *in vitro* phosphorylation sites in recombinant wheat eIF5 at S209, T240, and S451. Various mutants containing alanine or glutamate substitutions at the three phosphorylation sites have been cloned and expressed. Amino acid substitution at these residues reduces or eliminates the *in vitro* phosphorylation of eIF5 by CK2 (Figure 3.3). Wild-type eIF5 (Figure 3.3, lanes 1 & 4), which contains 3 phosphorylation sites, is more heavily phosphorylated than eIF5 S451A (lanes 2 & 5). The triple alanine mutant eIF5AAA (lanes 3 & 6) is no longer phosphorylated by CK2 *in vitro*.

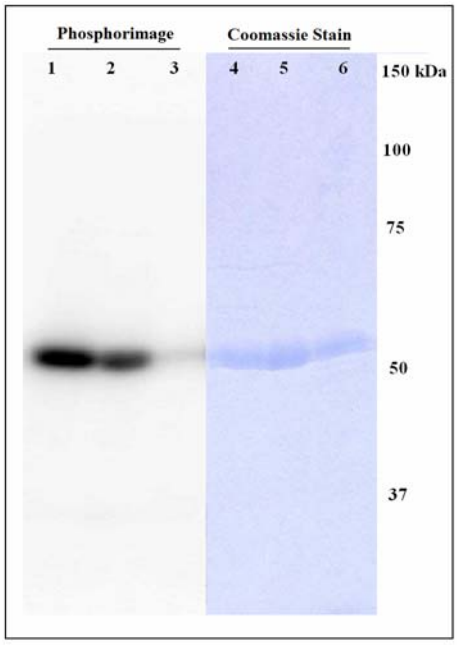



**Figure 3.3. Phosphorylation of eIF5 and mutant eIF5 by CK2**

Recombinant wheat eIF5, eIF5 S451A, and eIF5AAA were all incubated in the presence of CK2. Each 20 µl reaction contained 5 µg of eIF5, 0.5 µg of recombinant CK2α, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~200 cpm/pmol), and 100 mM KCl. Reactions were separated by SDS-PAGE, stained with Coomassie Blue and analyzed by phosphorimaging. Lanes 1, 2, & 3 are from phosphorimaging; Lanes 4, 5, & 6 are Coomassie stained gel.

Native wheat eIF5 was purified from wheat germ in the presence of the protein phosphatase inhibitor calyculin A. The linear MS spectra produced from the sample following IMAC enrichment contains a peak representing the phosphorylated C-terminal tryptic fragment plus one phosphate (+80 Da). This peak matches the spectra from recombinant wheat eIF5 that was phosphorylated *in vitro* by CK2 (Figure 3.4). Mass spectroscopic analysis of wheat germ eIF5 revealed two distinct isoforms of eIF5 (TC234451 and TC234445), and both were nearly 100% phosphorylated at the C-

terminal residue corresponding to S451 (Figure 3.5). There is also evidence for the partial phosphorylation of both S209 and T240 in this preparation. There is a strong signal in the endogenous sample representing peptide *205*GAGG**S**DEEHVSSPR*218* plus one phosphate, but unfortunately there is only a neutral loss of 98 Da with no other fragment ions in the spectra. Thus it is possible that this could also represent phosphorylation of either S215 or S216; however, only one residue is phosphorylated on the peptide. A weaker signal for peptide *220*DADFAAAADGDDDDDDDVQWA**T**DTSAEAAR*248* plus one phosphate was also detected, but again there was only a neutral loss of 98 Da with no other fragment ions. While our analysis could not conclusively identify S209 and T240 as endogenous phosphorylation sites, there is some suggestion that these residues are partially phosphorylated in wheat germ extracts. Based on the low stoichiometry of phosphorylation at these sites it is difficult to resolve the identity of the phosphorylated residues in these two fragments. It is possible that the use of other phosphatase inhibitors would improve the stoichiometry of phosphorylation at S209 and T240. No phosphorylation sites could be detected in native wheat eIF5 when purified in the absence of the phosphatase inhibitor calyculin A.

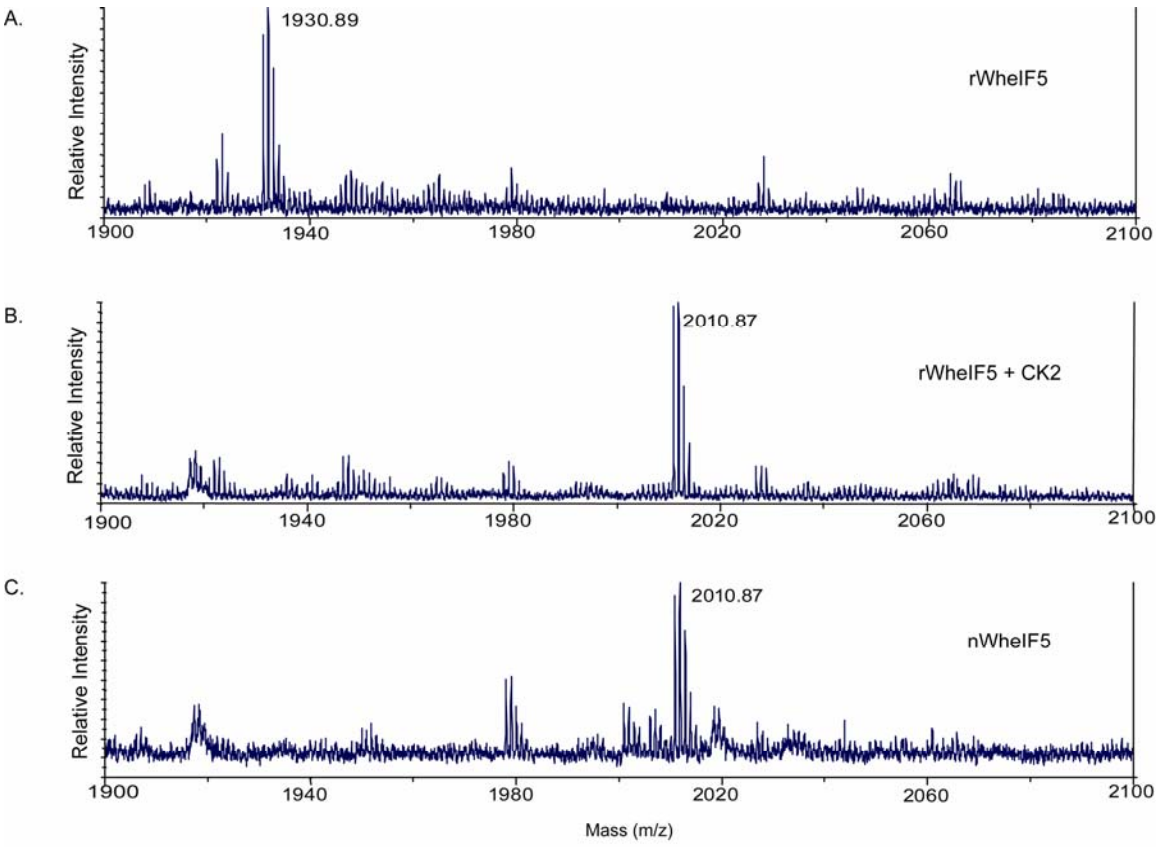



**Figure 3.4. Wheat eIF5 is a phosphoprotein**

MS analysis of eIF5 peptides following IMAC enrichment. MALDI-TOF spectrum of peptides from recombinant wheat eIF5 (A), recombinant wheat eIF5 phosphorylated by CK2 *in vitro* (B), and native wheat eIF5 (C). A shift in peptide mass from 1930.89 to 2010.87 by +80 Da is shown. This peptide sequence corresponds to the wheat eIF5 C-terminal tryptic peptide *438*NAKPVVEWLESAE**S**DEE*454*. This peptide exhibits the expected mass of 1930.89 Da in recombinant wheat eIF5 (A). Following *in vitro* phosphorylation by CK2 (B), this peptide shifts by +80 Da to 2010.87, representing the addition of one phosphate. Native wheat eIF5 purified in the presence of phosphatase inhibitors exhibits a similar peak at 2010.87 and the absence of any peak at 1930.89, suggesting it is fully phosphorylated.

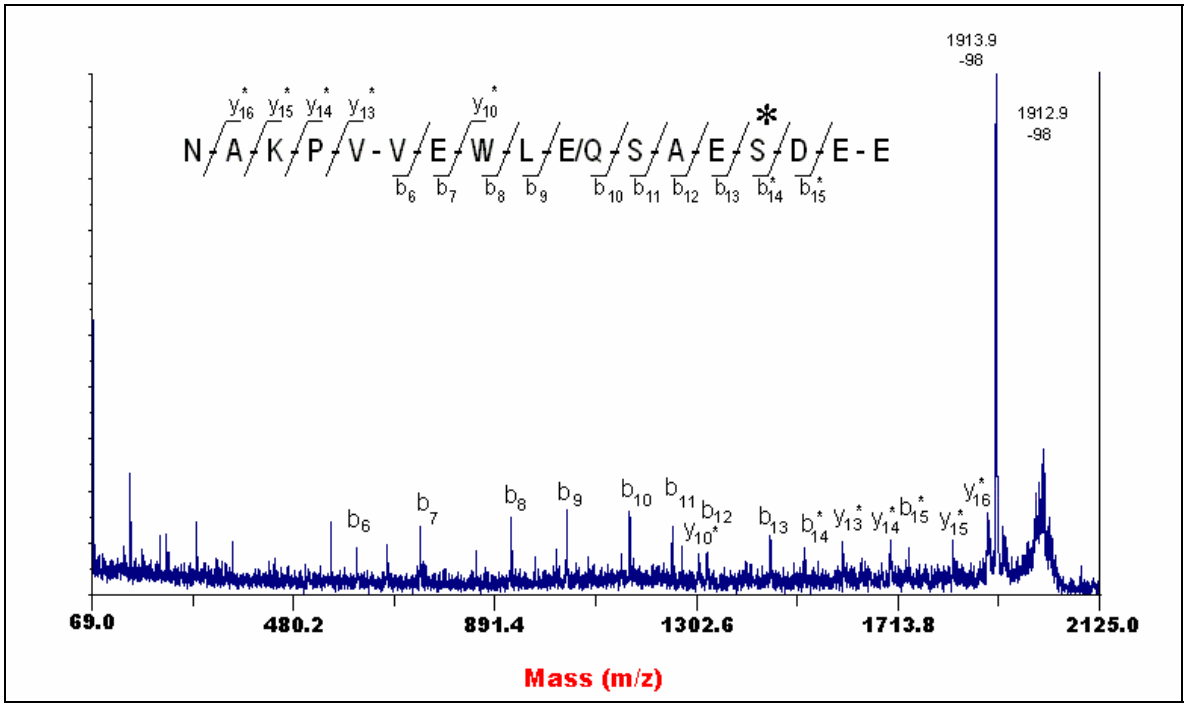



**Figure 3.5. Native wheat eIF5 phosphorylation site identification**

Native wheat eIF5 was analyzed by tandem MS following IMAC sample enrichment. This sample contained two distinct eIF5 isoforms, represented by the MS/MS of 2010.9 and 2011.9 that correspond to peptide sequences NAKPVVEWLESAES(P$_i$)DEE (m/z 2011.9) and NAKPVVEWLQSAES(P$_i$)DEE (m/z 2010.9). Fragment ion peaks from the two isoforms show monoisotopic doublets representative of peptide doublet shifted by 1 Da. Fragment ions shifted by the addition of a phosphate are indicated with an asterisk (*). Based on the shift of b- and y-ions, both peptides are phosphorylated on the residue corresponding to S451.

**eIF4B**. Wheat eIF4B has 4 predicted CK2 consensus sites. We have identified 2 *in vitro* CK2 phosphorylation sites in recombinant wheat eIF4B; however, neither of these were the predicted consensus sites. Wheat eIF4B is observed fully phosphorylated by CK2 on S140 *in vitro*. There is also a phosphorylation site on peptide *234*DSPGPSDS**S**DR*243*. An analysis of MS/MS data points to S241 as the

phosphorylated residue; however, there is some suggestion that the more favorable CK2 consensus site S239, while somewhat less likely, may alternatively be phosphorylated. However, the peptide was not observed in a doubly phosphorylated form. Both of these sites are conserved in rice eIF4B; however, neither is conserved in the protein sequences of *A. thaliana* eIF4B1 or *A. thaliana* eIF4B2 (see Figure 3.11 in Appendix). This suggest that there may be some difference in the phosphorylation of monocots and dicots. Unfortunately, no spectra were obtained for the tryptic fragment containing the most favorable CK2 consensus site (S106), thus it is possible that this residue is also phosphorylated *in vitro*. Studies are currently underway to evaluate the phosphorylation of recombinant *A. thaliana* eIF4B by CK2.

**nCBP**. Recombinant *A. thaliana* nCBP (novel cap-binding protein) contains a single CK2 consensus site in its N-terminal region (S42), and during *in vitro* assays containing [$\gamma$-$^{32}$P]ATP the native protein appears to be phosphorylated by CK2 (observation from this lab). Mass spectroscopic analysis of the phosphopeptides produced from *A. thaliana* nCBP was inconclusive as to the identity of the phosphorylated residue. Recombinant wheat nCBP has since been cloned and expressed in E. coli, and further analysis of CK2 phosphorylation of nCBP is in progress.

### 3.3.2. Implications of CK2 Phosphorylation

The differential phosphorylation of recombinant wheat initiation factors by plant CK2 isoforms has been demonstrated *in vitro* (Chapter 2); however the effect CK2 phosphorylation has on each factor is unknown. Identification of the specific residues modified by CK2 phosphorylation could provide some insight into the possible role CK2

plays in translation. In this study, 20 *in vitro* CK2 phosphorylation sites have been identified in recombinant wheat eIF2α, eIF2β, eIF3c, eIF4B, and eIF5 (Table 3.1). This is the first study to extensively identify the CK2 phosphorylation sites in initiation factors from a single system. A majority of these sites were located in the N- and C-termini of proteins, which generally have less structure, and are therefore good targets for phosphorylation and/or participating in protein-protein interactions. A native preparation of eIF5 was also analyzed and native wheat eIF5 was determined to be a phosphoprotein containing at least 3 phosphates. The C-terminal CK2 site (S451) of native eIF5 was completely phosphorylated, and tryptic fragments containing the other two sites (S209, T240) also appear to be partially phosphorylated. If phosphorylation sites were critical to function, one would expect the residues to be conserved in other plant species. This is indeed the case for a number of the CK2 phosphorylation sites identified in this research. The mapping of CK2 phosphorylation sites represents an initial step in elucidating the role phosphorylation plays in regulation of plant translation initiation factor activity. Identification of phosphorylation sites is a prerequisite of the generation of mutant factors for biochemical studies and the development of phosphopeptide specific antibodies.

These experiments are particularly important in determining the potential role CK2 phosphorylation plays in the interaction of factors within the MFC (multifactor complex), which consists of eIF3/eIF1/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$. It is striking that 3 of the 4 factors involved are all substrates for CK2. Wheat eIF1 does not appear to be phosphorylated by CK2, nor does it contain any predicted phosphorylation sites for CK2 or any other protein kinases (www.scansite.mit.edu). Of particular interest is the heavily phosphorylated N-terminus of eIF3c and the C-terminus of eIF5, which both serve as

hubs for multifactor complex assembly (Valasek et al., 2004; Yamamoto et al., 2005). Binding studies from yeast suggest that acidic residues in the N-terminus of eIF3c interact with basic residues in eIF1 and eIF5 (Valasek et al., 2004); and an acidic/aromatic domain in the C-terminus of eIF5 interacts with the K-boxes of eIF2β (Valasek et al., 2002). These predicted binding regions appear to be conserved in plant initiation factors, and a number of the CK2 phosphorylation sites identified in this study are located in these critical binding domains. It is suspected that CK2 phosphorylation of these domains could modify interactions within the multifactor complex.

Phosphorylation of the wheat eIF2α C-terminus by CK2 is not unique, as three CK2 phosphorylation sites have been previously identified in the yeast eIF2α (SUI2 gene product) C-terminus (Feng et al., 1994). No similar phosphorylation of eIF2α by CK2 is seen in mammalian cells. However, this phosphorylation site does appear to be conserved across plants, suggesting that they are functionally relevant (Figure 3.6). Mutation of the SUI2 C-terminus to alter its 3 CK2 sites to alanines did not cause a detectable phenotype; however, when combined with other mutations that reduced the efficiency of nucleotide exchange (GCN2, GCN3, GDC7), the SUI2 alanine mutations did exacerbate growth defects. This would suggest that the phosphorylation of eIF2α in yeast may be required for optimum eIF2 activity, and it is possible that S318 phosphorylation in wheat may play a similar role (Figure 3.6).

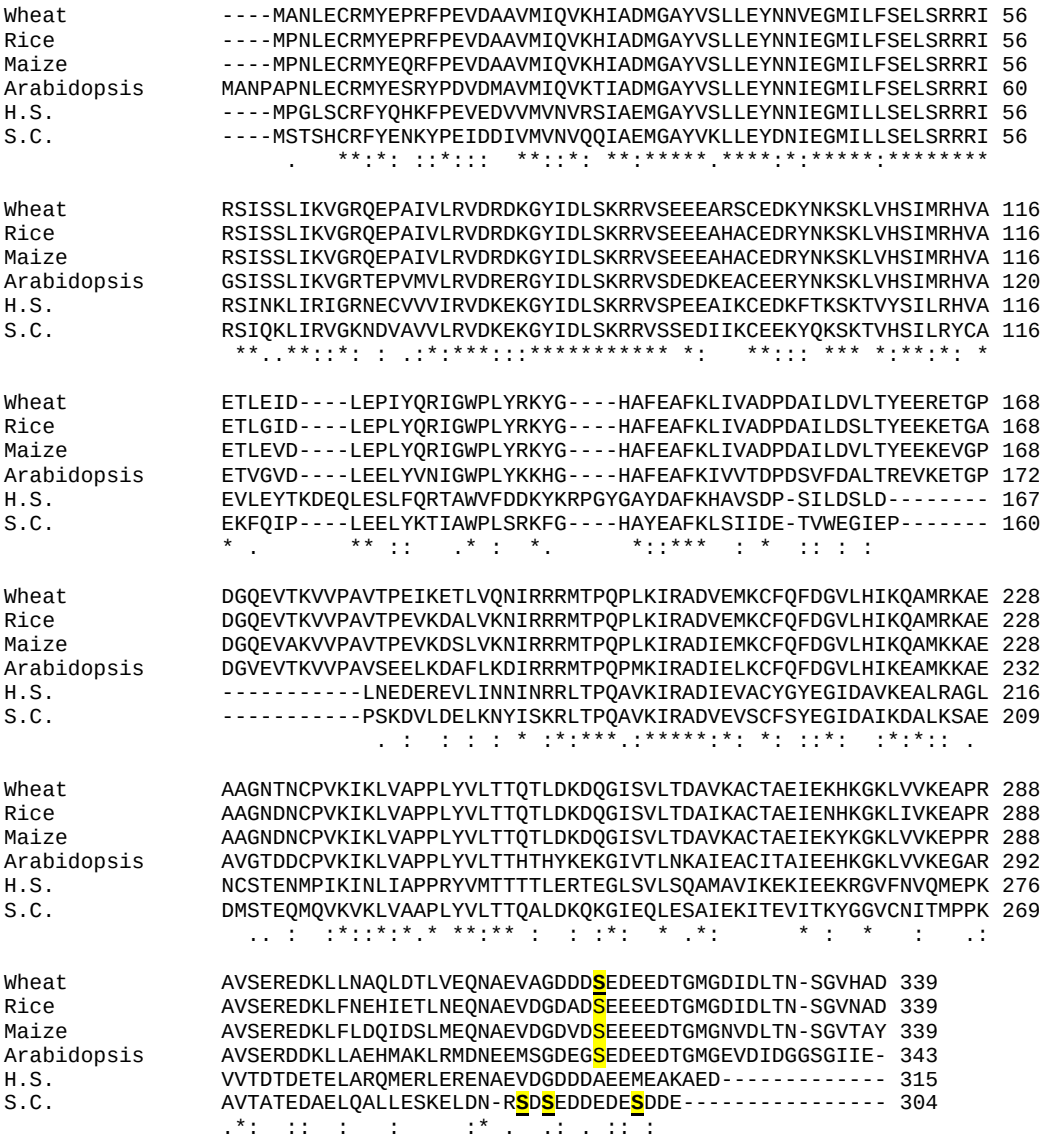



**Figure 3.6. Alignment of eIF2α protein sequences**

Multiple sequence alignment of eIF2α was performed using Clustal 2.0.2. Shown are the sequences for the wheat eIF2α (*TC263298*), rice (*EAY89622*), maize (*TC318839*), *A. thaliana* (*AAK29673*), human (*P05198*), and *S. cerevisiae* (*EDN63334*). Wheat eIF2α is phosphorylated *in vitro* by CK2 phosphorylation at S318, and this phosphorylation site appears to be conserved in plants. The CK2 phosphorylation site identified in this study is indicated in bold and underline. CK2 phosphorylation sites identified in yeast eIF2α were previously identified *in vivo* (Feng et al., 1994).

Due to its location in C-terminal domain of eIF2α, it is possible that CK2 phosphorylation of S318 may play a role in regulating ternary complex formation. The C-terminus of archaeal initiation factor 2α (aIF2α) is the predicted binding domain for aIF2γ and its binding site on aIF2γ is located in close proximity to the Met-tRNA$_i^{Met}$ binding (Yatime et al., 2004). Due to the location of its binding site, there is speculation that the C-terminus of aIF2α contributes to complex formation between Met-tRNA$_i^{Met}$ and aIF2 through a direct interaction with the polynucleotide or by triggering a conformational adjustment of the tRNA binding site on the γ subunit (Yatime et al., 2004). There is a high degree of conservation both among eukaryotes and with archaebacteria (Kyrpides and Woese, 1998) and a similar interaction likely takes place in plants.

The phosphorylation site identified here (S318) in wheat eIF2α is distinct from the S51 involved in regulation of mammalian eIF2 guanine nucleotide exchange. It is not clear that plants use phosphorylation of eIF2α for regulation since comparable kinases (*e.g.* PKR, HRI) have yet to be isolated from plants (Browning, 2004). There is no site similar to S318 in mammalian eIF2α (Figure 3.6), and mammalian CK2 does not appear to phosphorylate eIF2α (Llorens et al., 2005b).

Wheat eIF2β was phosphorylated *in vitro* in the presence of the CK2 holoenzyme on T52/S54 (residues could not be distinguished in the MS/MS) and T85. CK2 phosphorylation of the eIF2β N-terminal domain is seen in mammalian cells, yet this phosphorylation does not appear to be conserved in yeast (Llorens et al., 2005a; Llorens et al., 2005b). The N-terminal domain of eIF2β also contains a series of lysine blocks that participate in binding to eIF5, eIF2Bε, and mRNA (Asano et al., 1999). In

mammalian cells, the proper phosphorylation of eIF2β by CK2 in is required for its function, as overexpression of a non-phosphorylatable mutant of eIF2β leads to a reduction in the rate of protein synthesis stimulated by serum (Llorens et al., 2005a). The primary site of eIF2β phosphorylation in mammalian cells was identified as S2, with a secondary (partial) phosphorylation occurring on S67 (Llorens et al., 2005a). It is not known if the wheat eIF2β phosphorylation sites are comparable. Multiple sequence alignment of wheat and human eIF2β amino acid sequences aligns T52/S54 with the mammalian S67 site; however the S2 site in mammalian eIF2β is not conserved in plants or yeast, nor is there conservation of the wheat T85 site across all plants (Figure 3.7). Interestingly, the N-terminal domain of plant eIF2β only contains 2 lysine blocks (unlike mammalian and yeast which contain 3), thus some variance in the function and regulation of this domain is likely.

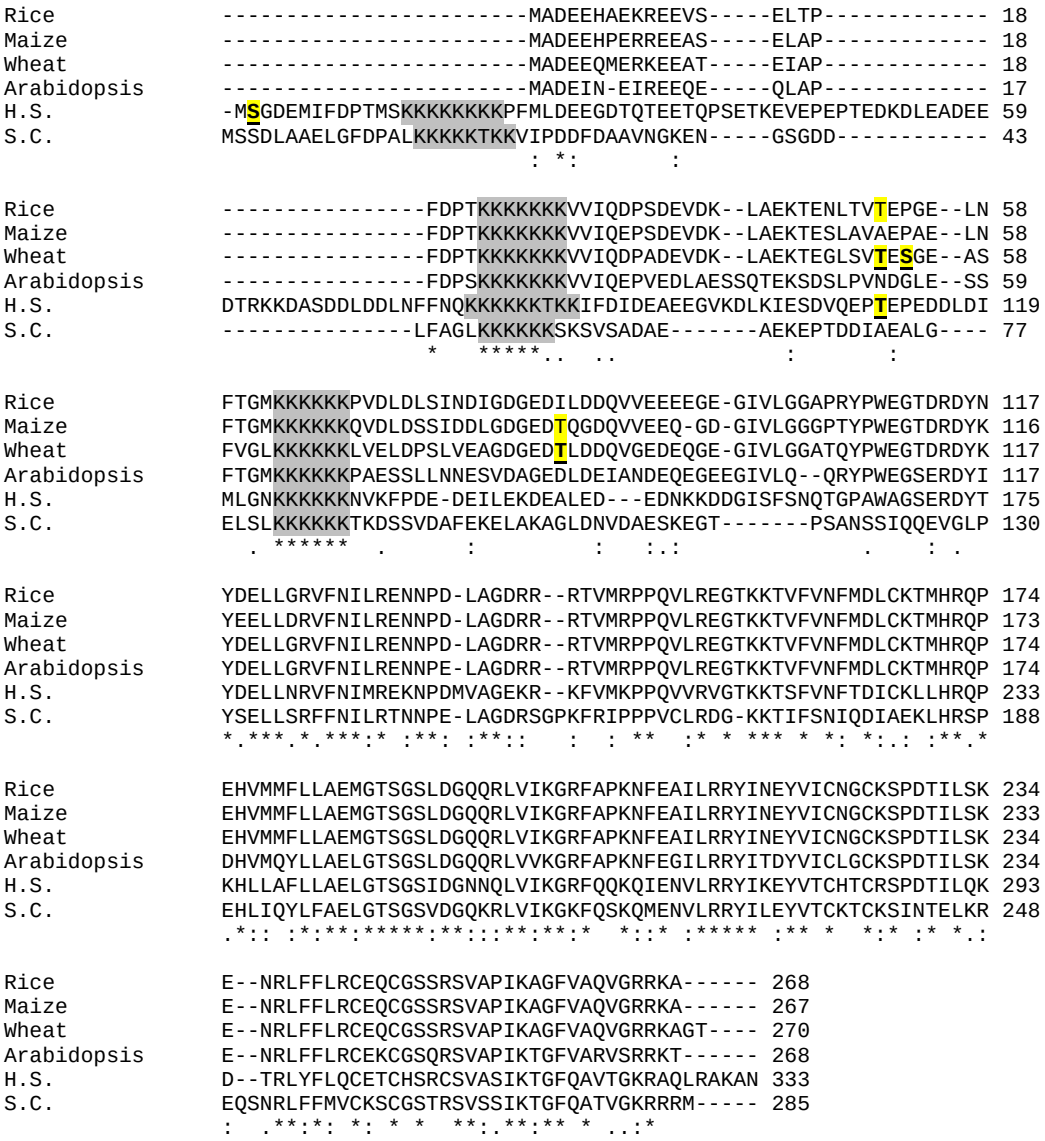



**Figure 3.7. Alignment of eIF2β protein sequences**

Multiple sequence alignment of eIF2β was performed using Clustal 2.0.2. Shown are the sequences for the wheat (*0Z4473*), rice (*Os07g0681000*), maize (*TC318899*), *A. thaliana* (*AAK29672*), human (*NP_003899*), and *S. cerevisiae* (*SUI3*) eIF2β. Wheat eIF2β is phosphorylated *in vitro* by CK2 phosphorylation on two residues. The phosphorylation of T52 or S54 could not be distinguished in the MS/MS; however, T85 was unambiguously identified. These phosphorylation sites do not appear to be conserved in all plants. The CK2 phosphorylation site identified in this study is indicated in bold and underline. CK2 phosphorylation sites indentified in mammalian eIF2β were previously identified *in vivo* (Llorens et al., 2005b). Indicated in grey are the K-boxes of eIF2β which participate in binding to eIF2B, eIF5 and mRNA (Asano et al., 1999).

The *in vitro* phosphorylation of eIF3c by CK2 is extensive, as 12 sites have been identified using recombinant wheat eIF3c-NTD. A large number of these sites are conserved across plants (Figure 3.8). This large amount of modification could alter the function of eIF3, which includes binding to the 40S ribosomal subunit, promoting binding of mRNA to the 40S ribosome, and facilitation of 40S and 60S ribosome dissociation (Hinnebusch, 2006). The N-terminus of wheat eIF3c is highly acidic; the pI of the first 224 N-terminal residues is 4.36. Acidic residues in the N-terminus of yeast eIF3c (NIP1) have been shown to play an important role in binding to both eIF1 and eIF5 (Valasek et al., 2004). Multiple sequence alignment of eIF3c sequences aligns a number of the CK2 phosphorylation sites identified in wheat with these domains in yeast that have been shown to mediate binding to eIF1 and eIF5 (Figure 3.8). The addition of phosphates to this region would further acidify these domains and could alter their ability to bind to eIF1 and/or eIF5. Yeast eIF1 and eIF5 contain basic domains with high levels of lysine and arginine residues termed KR (Reibarkh et al., 2008) and Area II (Yamamoto et al., 2005), which play a critical role in their association with yeast eIF3c (NIP1).

Interestingly, mammalian eIF3 was the first physiological substrate of CK2 identified in 1976 (Pinna, 1994). Mapping the phosphorylation sites in this region has likely been delayed by the complexity of phosphorylation and the difficulty in working with highly acidic peptides using mass spectrometry. This phenomenon is clearly demonstrated in the MS analysis of eIF3c-NTD seen in Figure 3.6. Recently, a hexaphosphorylated tryptic peptide was identified *in vivo* from the N-terminal region of the mammalian eIF3c (Tokuda et al., 2006). The potential kinases responsible for their

phosphorylation were predicted by Scansite (www.scansite.mit.edu) to be the acidophilic serine kinases, CK1 or CK2 (Damoc et al., 2006). It is likely that the findings presented here represent a similar and possibly more dramatic phosphorylation event in plants. The recent public release of the Arabidopsis Protein Phosphorylation Site (*PhosPhAt* 2.1*)* database provided *in vivo* evidence of eIF3c N-terminus phosphorylation at the consensus CK2 site S40 (Heazlewood et al., 2008). The phosphorylation of *A. thaliana* eIF3c at S40 corresponds to wheat eIF3c S53 (see Figure 3.8 and Appendix Figure 3.12), which was observed to be phosphorylated by CK2 *in vitro*.

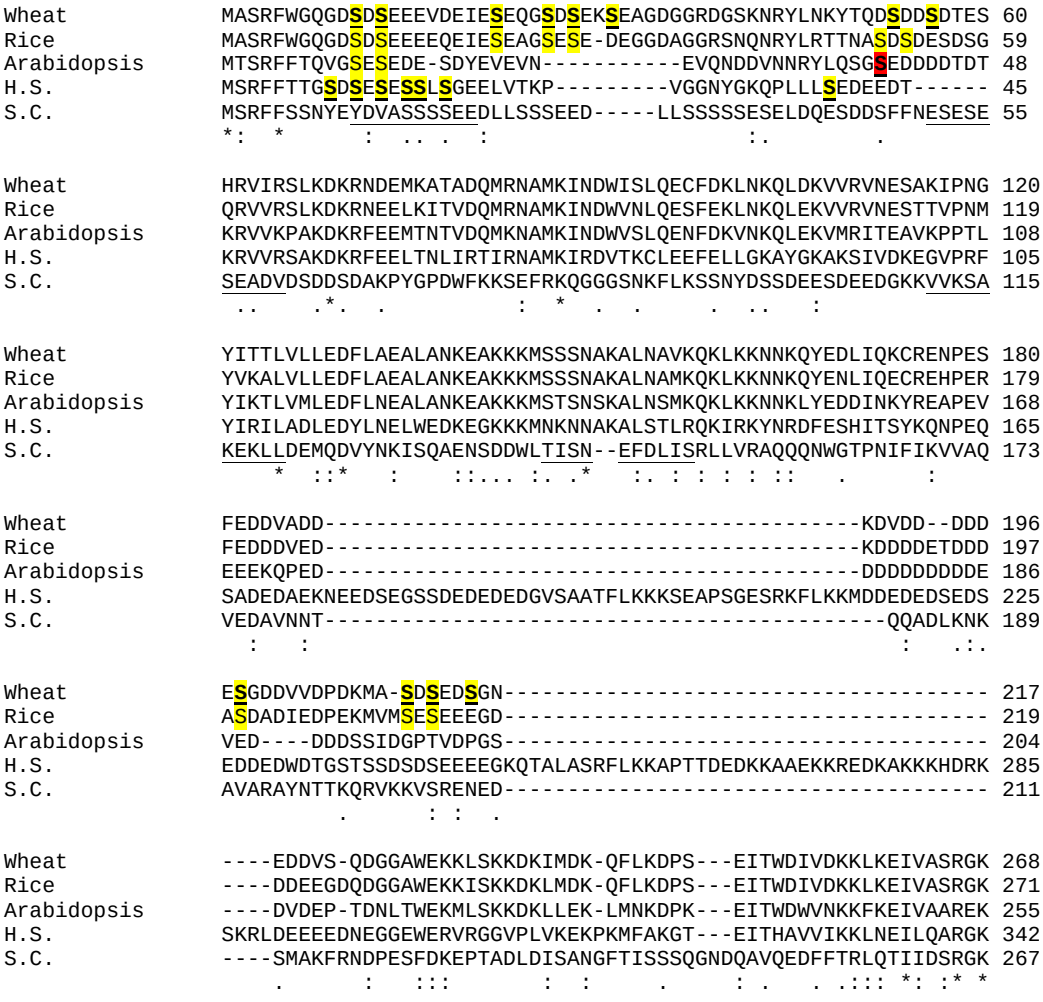



**Figure 3.8. Alignment of eIF3c N-terminal amino acids**

Multiple sequence alignment of eIF3c N-terminal residues was performed using Clustal 2.0.2. Shown are the sequences for the N-terminus of wheat eIF3c (*TC235391*), rice (*EAZ38556*), *A. thaliana* (*AAC83464*), *S. cerevisiae* (*END64250*), and human (*AAD03462*). CK2 phosphorylation sites are indicated in bold and underline. 12 CK2 phosphorylation sites were identified in the N-terminus of recombinant wheat eIF3c-NTD. Conserved consensus CK2 sites in the N-terminus eIF3c sequences are indicated. The *in vivo* phosphorylation of the eIF3c N-terminus at consensus CK2 site has been observed by large-scale phosphoproteonomic analysis in *A. thaliana* (indicated in red, (Heazlewood et al., 2008) and human (Damoc et al., 2006). Segments in the N-terminus of yeast eIF3c that were implicated in binding eIF1 and eIF5 are indicated with a solid line (Valasek et al., 2004). For an alignment of plant sequences only see Appendix Figure 3.12.

The sites of eIF5 phosphorylation by CK2 have been previously reported *in vitro* and *in vivo* in both yeast (Maiti et al., 2003) and mammals (Majumdar et al., 2002); however, it was not known if similar phosphorylation sites existed in plants. Three *in vitro* CK2 phosphorylation sites have been identified in wheat eIF5 at S209, T240, and S451. Our analysis of native wheat eIF5 revealed that it is multiply phosphorylated and S451 is almost entirely phosphorylated on two distinct isoforms of eIF5. This analysis also revealed phosphopeptides representing the tryptic fragments containing S209 and T240; yet the specific residues of phosphorylation could not be identified in the spectra. All three of the CK2 phosphorylation sites in eIF5 are conserved across plants, and similar CK2 phosphorylation sites exist in the C-terminus of yeast and mammalian protein sequences (Figure 3.9).

```
TC234451        MALQNIGASNKDDAFYRYKMPRMLTKIEGRGNGIKTNIVNMVDIAKALARPASYTTKYFG 60
TC234445        MALQNIGASNKDDAFYRYKMPRMLTKIEGRGNGIKTNIVNMVDIAKALARPASYTTKYFG 60
Maize           MALQNIGASNRDDAFYRYKMPRMITKIEGRGNGIKTNVVNMVDIAKALARPASYTTKYFG 60
Rice            MALQNIGASNRDDAFYRYKMPRMITKIEGRGNGIKTNIVNMVDIAKALARPASYTTKYFG 60
Arabidopsis     MALQNIGASNRNDAFYRYKMPKMVTKTEGKGNGIKTNIINNVEIAKALARPPSYTTKYFG 60
S.C.            MSIN--ICRDNHDPFYRYKMPPIQAKVEGRGNGIKTAVLNVADISHALNRPAPYIVKYFG 58
H.S.            MSVN--VNRSVSDQFYRYKMPRLIAKVEGKGNGIKTVIVNMVDVAKALNRPPTYPTKYFG 58
                *:::     .  * ******* : :* **:****** ::* .::::** **..* .****

TC234451        CELGAQSKFDEKTGISLVNGAHDTLKLAGLLENFIKKYVQCYGCGNPETEVLIS-KKEMI 119
TC234445        CELGAQSKFDEKTGISLVNGAHDTSKLAGLLENFIKKYVQCYGCGNPETEVLIS-KKEMI 119
Maize           CELGAQSKFDEKTGISLVNGAHDTAKLAGLLEVFIKKYVQCYGCGNPETEILIS-KTQMI 119
Rice            CELGAQSKFDEKTGTSLVNGAHDTAKLAGLLENFIKKYVQCYGCGNPETEVLIS-KAQMI 119
Arabidopsis     CELGAQSKFDEKTGTSLVNGAHNTSKLAGLLENFIKKFVQCYGCGNPETEIIIT-KTQMV 119
S.C.            FELGAQTSISVDKDRYLVNGVHEPAKLQDVLDGFINKFVLCGSCKNPETEIIIT-KDNDL 117
H.S.            CGLGAQTQFDVKNDRYIVNGSHEANKLQDMLDGFIKKFVLCPECENPETDLHVNPKKQTI 118
                  ****:.:. ...  :*** *:. ** .:*: **:*:* *  * ****:: :. * : :

TC234451        TLKCAACGFLSDVDMRDKLTTFILKNPPEAKKGG-KDKKAMRRAEKERLKEGEAADEEMK 178
TC234445        TLKCAACGFLSDVDMRDKLTTFILKNPPEAKKGG-KDKKAMRRAEKERLKEGEAADEEMK 178
Maize           SLKCAACGFVSDVDMRDKLTTFILKNPPEQKKGG-KDKKAMRRAEKERLKEGEAADEEQK 178
Rice            TLKCAACGFVSDVDMRDKLTTFILKNPPEQKKGGGKDKKAMRRAEKERLKEGEAADEEMK 179
Arabidopsis     NLKCAACGFISEVDMRDKLTNFILKNPPEQKKVS-KDKKAMRKAEKERLKEGELADEEQR 178
S.C.            VRDCKACGKRTPMDLRHKLSSFILKNPP---DSVSGSKKKKKAATASANVRGGGLSIS-- 172
H.S.            GNSCKACGYRGMLDTHHKLCTFILKNPPENSDSGTGKKEKEKKNRKGKDKENGSVSSS-- 176
                  .* ***    :* :.** .*******   .    .*:  :        ..   . .

TC234451        KLKKDGKKKGASSKESTAKGVATKKKGAGGSDEEHVSSPRDADFAAAADGDDDDDDDVQW 238
TC234445        KLKKDGKKKGASSKESTAKGVATKKKGAGGSDEEHVSSPRDADFAAAADDDDDDDDDVQW 238
Maize           KLKKDAKKKGS--KDSTAKGLKKKATTATGSDEDHSSSPTRSHDGDKAAADDDDDD-VQW 235
Rice            KLKKEAKKKGASKESTSSKSGAGKKKAASGSDEDHSNSPTRSHDGDNVAADEDDDDDVQW 239
Arabidopsis     KLK--AKKKALS------NGKDSKTSKNHSSDED--ISPKHDENALEVDEDEDDDDGVEW 228
S.C.            ------------------DIAQGKSQNAPSDGTGSSTPQHHDEDEDELSRQIKAAASTLE 214
H.S.            ------------------ETP---PPPPPPNEINPPPHTMEEEEDDDWGEDTTEEAQRRR 215
                                   .          .          .      :

TC234451        ATDTSAEAARKRMEEQLSAATAEMVMLATEETEKKKKQAQHKDDGGANGTAKAEETSNGN 298
TC234445        ATDTSAEAARKRMEEQLSAATAEMVMLATEETEKKKKQAQHKEDG-ANGTSKAEETSNGK 297
Maize           QTDTSIEAAKQRMQEQLSAATAEMVMLSTEETEKKMKQPTHKDGS-TNGS--AKEIPNDK 292
Rice            QTDTSLEAAKQRMQEQLSAATAEMVMLSTEEPEKKKKHEALHKEGTSNGS------AKHV 293
Arabidopsis     QTDTSREAAEKRMMEQLSAKTAEMVMLSAMEVEEKKAPKSK-----SNGN---------V 274
S.C.            DIEVKDDEWAVDMSEEAIRARAKELEVNSELTQLDEY----------------------- 251
H.S.            MDEISDHAKVLTLSDDLERTIEERVNILFDFVKKKK----------------------- 251
                  : . .     : ::      : : :     : .

TC234451        QNGAKTTTPYDALVEEIKASLANAATAAQLKGLLSSSPLPLKDVMDALFEALFHGAAKGF 358
TC234445        QNGAKAT-PYDELVEEIKASLANAATAAQLKGVLSSSTLPLKDVMDALFEALFHGAGKGF 356
Maize           PAVTKPS-PYEELIGDIKASLGSAPTPSQLKAVLASSTLPPQDVMNAPLEALFGGVGKGF 351
Rice            VEEAKSS-PYDDLVKEMKDNLSKGATAVQLKGLMTSSALPPQDAMNALFDALFGGLSKGF 352
Arabidopsis     VKTENPPPQEKNLVQDMKEYLKKGSPISALKSFISSLSEPPQDIMDALFNALFDGVGKGF 334
S.C.            --------GEWILEQAGEDKENLPSDVELYKKAAELDVLNDPKIGCVLAQCLFDEDIVNE 303
H.S.            -------------EEGVID----SSDKEIVAEAERLDVKAMGPL--VLTEVLFNEKIREQ 292
                                     .                     .  : **

TC234451        AKDVVKNRKYLAAAVP--DEAAQMLLLQSIEAFCGKC-SAEALKELPVVLKALYDGDVLE 415
TC234445        AKDVVKNKKYLAAAVP--DEAAQMLLLQSIEAFCSKC-SAEALKELPVVLKALYDGDLLE 413
Maize           TKEVVKNKKYLAVAVP--DEGAQTLLVQAIEAFGGKC-NPEALKEVPVVLKALYDGDILD 408
Rice            AKEVVKKKKFLAAAVP--DEASQMVLLQALVAFGAKS-SPEAVKEVPIVLKALYDGDILD 409
Arabidopsis     AKEVTKKKNYLAAAATMQEDGSQMHLLNSIGTFCGKNGNEEALKEVALVLKALYDQDIIE 394
S.C.            IAEHNAFFTKILVTP-----EYEKNFMGGIERFLGLE-HKDLIPLLPKILVQLYNNDIIS 357
H.S.            IKEYRRHFLRFCHNNK----KAQRYLLHGLECVVAMH-QAQLISKIPHILKEMYDADLLE 347
                  :       :          :  :: .:  . .     : :  :. :*  :*: *::.
```

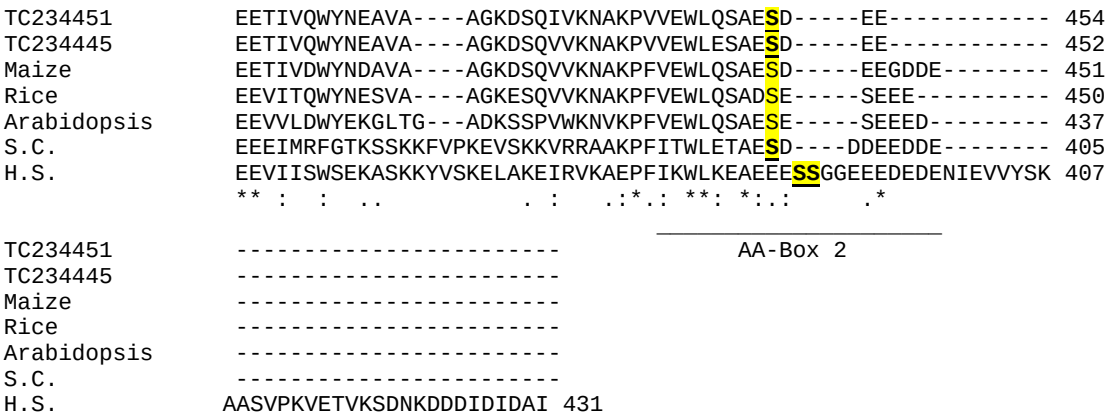



**Figure 3.9. Alignment of eIF5 protein sequences**

Multiple sequence alignment of eIF5 was performed using Clustal 2.0.2. Shown are sequences of wheat (*TC234451* and *TC234445*), maize (*CAA10616*), rice (*EAZ02236*), *A. thaliana* (*NP177907*), *S. cerevisiae* (P38431), and human (CAG32993) eIF5 proteins. Three *in vitro* CK2 phosphorylation sites were identified in recombinant wheat eIF5 (*TC234451*) at S209, T240, and S451. All three of these sites are conserved in plants. CK2 phosphorylation sites are indicated in bold and underline. CK2 phosphorylation sites indicated in *S. cerevisiae* and Human eIF5 were previously identified (Majumdar et al., 2002; Maiti et al., 2003). The acidic/aromatic-box 2 (AA-Box 2) responsible for the interaction of yeast and mammalian eIF5 with the K-boxes of eIF2β is indicated with a solid line.

Yeast eIF5 binds to eIF2β through an AA-box (acidic/aromatic-box) in its C-terminus, and this interaction is required for MFC formation (Das and Maitra, 2000). The phosphorylation of the C-terminal AA-boxes of eIF5 appears to be conserved in all systems analyzed to date. Mutation of human eIF5 AA-box residues S389 and S390 to alanine perturbs cell cycle progression and reduces the formation of the normal translation initiation complex *in vivo* (Homma et al., 2005). It is not known if phosphorylation of a single residue in the C-terminus, as seen here in wheat and

previously reported in yeast (Maiti et al., 2003), plays an equally important role in regulating binding to eIF2β. It is important to note that the C-terminus of human eIF5 also contains approximately 33 additional amino acids that are absent from plants and yeast (Figure 3.9).

### 3.4. CONCLUSIONS

The phosphorylation of initiation factors involved in the multifactor complex has not been well characterized in plants or other organisms. The results presented here represent a starting point for evaluating the role CK2 phosphorylation plays in regulating plant translation initiation. CK2 phosphorylates a number of key initiation factors involved in the formation of the multifactor complex (eIF2, eIF3, and eIF5). Upon phosphorylation site identification it became apparent that a number of the key domains involved in factor interaction were the targets of CK2. Of particular interest is the phosphorylation of eIF3c and eIF5, which serve as key hubs of multifactor complex assembly in yeast. It is hypothesized that the phosphorylation of multiple wheat initiation factors by CK2 could play a role in regulating multifactor complex formation (Figure 3.10).

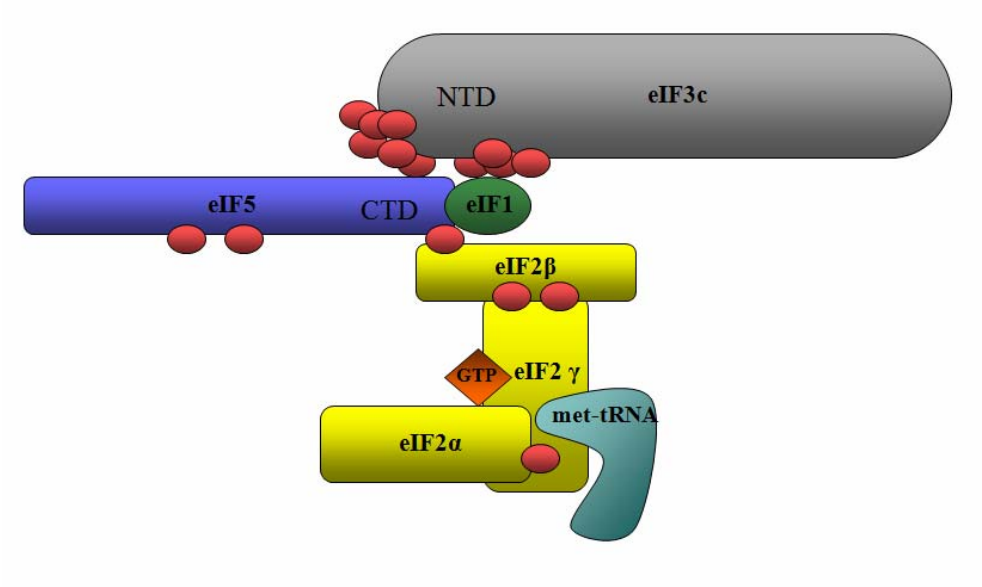



**Figure 3.10. Hypothesized model for CK2 phosphorylation in wheat MFC**

Multiple CK2 phosphorylation sites have been identified in wheat initiation factors involved in the formation of the multifactor complex (MFC), which consists of eIF1, eIF3, eIF5, and the eIF2 ternary complex. Based on the location of these phosphorylation sites it is hypothesized that phosphorylation of wheat eIF3c and eIF5 by CK2 may regulate formation of the multifactor complex. For simplicity, only the eIF3c subunit of eIF3 is shown in this model, as eIF3 has 13 subunits. Using mass spectrometry, 12 *in vitro* CK2 phosphorylation sites have been identified in key binding domains in the N-terminus of eIF3c. Three *in vitro* CK2 sites were identified in wheat eIF5 at S209, T240, and S451; and native wheat eIF5 is phosphorylated on S451, and at least two other residues. Phosphorylation sites identified in this research are indicated as red spheres. The interactions of subunits depicted are based on studies in yeast, where the N-terminus of yeast eIF3c is known to mediate critical interactions with both eIF1 and eIF5 (Valasek et al., 2004), and the C-terminal domain of yeast eIF5 binds eIF1, the eIF2β N-terminus, and eIF3c N-terminus (Asano et al., 2001b). Due to the conservation of these binding domains, it is suspected that similar interactions occur in plants, and that these interactions could be mediated by the CK2 phosphorylation identified in this research.

# Chapter 4: CK2 Enhances the Formation of Plant Multifactor Complexes *in vitro*

## 4.1. INTRODUCTION

Translation initiation is a critical, rate-limiting step in eukaryotic gene expression, with a key step early in the pathway being the assembly of the 43S pre-initiation complex (Hershey and Merrick, 2000). This 43S pre-initiation complex is comprised of the small 40S ribosomal subunit, eIF1, eIF1A, the eIF2/GTP/Met-tRNA$_i^{Met}$ ternary complex, eIF3, and eIF5. Biochemical data suggests that a multifactor complex (MFC) consisting of yeast eIF3/eIF1/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$ exists free of the ribosome allowing these factors to be simultaneously loaded onto the 40S ribosomal subunit (Asano et al., 2000). Mutations that disrupt the interaction of factors within this complex result in substantial reductions in translation initiation (Asano et al., 2000; Reibarkh et al., 2008). The 43S pre-initiation complex is recruited to eIF4F and associated factors bound to mRNA, such that the complex can scan along the 5' untranslated region (UTR) for the proper AUG initiation codon, which will base pair with the anitcodon of the Met-tRNA$_i^{Met}$ (Kozak, 1978; Pestova et al., 1998). Initiation factors eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, and eIF4F have all been shown to be critical in the scanning process (Pestova et al., 2007). Once base pairing has taken place, GTP hydrolysis by eIF2 is triggered by its GTPase-activating protein (GAP), eIF5 (Das et al., 2001). eIF1 regulates both the GAP activity of eIF5 and the release of the $P_i$ generated by eIF2-GTP hydrolysis (Valasek et al., 2004). This process presumably culminates in the displacement of factors from the complex and joining of the 60S ribosomal subunit to form a functional 80S ribosome

with the Met-tRNA$_i$ in the ribosomal P site. The activities of initiation factors from many organisms are regulated through phosphorylation (Hershey, 1989; Proud, 1992; Flynn et al., 1997; Patel et al., 2002; Gingras et al., 2004; Proud, 2007). Overall however, the phosphorylation of initiation factors involved in the multifactor complex has not been fully characterized in plants or other organisms.

Using a variety of *in vitro* binding assays, two-hybrid analysis, and *in vivo* purification of affinity tagged subunits, an extensive analysis of the subunit interactions within the multifactor complex (MFC) has resulted in a provisional model of the yeast MFC (summarized in (Hinnebusch et al., 2007). The yeast eIF3c (NIP1) N-terminal domain plays a particularly important role in binding to other initiation factors in the context of the multifactor complex. The eIF3c N-terminal domain was found to bind directly to both eIF1 and eIF5 (Asano et al., 1998; Phan et al., 1998), and it is suspected that this binding coordinates the interaction between eIF1 and eIF5 that is responsible for the inhibition of GTP hydrolysis at non-AUG codons (Valasek et al., 2004). Meanwhile, the C-terminal domain of eIF5 simultaneously binds to both the N-terminus of eIF3c, the N-terminal K-boxes of eIF2β, and eIF1 (Asano et al., 2000; Yamamoto et al., 2005). This creates an indirect link between eIF3c and eIF2β, which is further supported by the direct interaction of eIF3a with eIF2β (Valasek et al., 2002) and the multiple interactions of eIF1 (Reibarkh et al., 2008). In yeast, eIF1 interacts directly with the N-terminal domains of both eIF2β and eIF3c, in addition to binding to the CTD of eIF5 (Valasek et al., 2004; Yamamoto et al., 2005; Reibarkh et al., 2008). It is not clear however, if all of these interactions are conserved in other eukaryotes, as NMR was unable to detect any

interaction between human eIF1 and a recombinant form of human eIF5 (Fletcher et al., 1999).

The predicted binding domains from yeast appear to be generally conserved in plant initiation factors when analyzed by multiple sequence alignment; however, evidence of multifactor complex interactions in plants only comes from the anecdotal observations of copurification. It has been observed that wheat eIF2 copurifies with eIF5, and eIF1 can be detected in purified samples of both wheat eIF3 and eIF5 by western analysis (MDDennis, unpublished data). This chapter represents a preliminary investigation into the interaction of plant eIFs 1, 2, 3, and 5 and provides biochemical evidence that a similar multifactor complex exists in plants that may be regulated by CK2 phosphorylation.

Due to the number of ionic interactions involved in multifactor complex (MFC) binding, it is suspected that post-translational modifications that alter amino acid charges, such as phosphorylation, may play an influential role in MFC formation. A large number of the interactions within the yeast MFC rely on charged clusters of amino acids. Glutamate and aspartate rich stretches in the N-terminal domain (NTD) of yeast eIF3c mediate the binding of eIF1 and eIF5 (Valasek et al., 2002). Conversely, basic domains containing high levels of lysine and arginine residues in eIF1 (Reibarkh et al., 2008) and eIF5 (Yamamoto et al., 2005) play a critical role in association with eIF3c. A positively charged binding domain in eIF5 also makes critical interactions with the acidic N-terminal tail of eIF1 (Reibarkh et al., 2008). Additionally, acidic residues in the C-terminus of eIF5 interact with the lysine rich surface of eIF1 (Reibarkh et al., 2008). Meanwhile, the AA-boxes (acidic/aromatic) of the eIF5 C-terminal domain have been

shown to bind to the lysine rich K-boxes of eIF2β (Asano et al., 1999). It is suspected that phosphorylation of these domains in yeast would serve as a means of regulating their interactions.

Emerging evidence suggests the formation of the eIF2/eIF5 complex in mammalian cells may be regulated via phosphorylation (Homma et al., 2005). The mammalian eIF5 C-terminal domain is multiply phosphorylated by CK2 in response to cell cycle progression (Homma and Homma, 2005). Phosphorylation of the eIF5 C-terminal HEAT domain appears to be conserved in mammals (Homma and Homma, 2005), yeast (Maiti et al., 2003), and plants (Chapter 3). However, in yeast and plants there is only a single CK2 phosphorylation site in the C-terminal AA-boxes. Expression of mutant mammalian eIF5 with alanine substitutions at CK2 phosphorylation sites reduced the formation of eIF5/eIF2 complexes *in vivo*, and resulted in significant reductions in growth (Homma et al., 2005). It is suspected that the phosphorylation of plant eIF5 by CK2 isoforms plays a similar role.

Plants have multiple forms of CK2 that are capable of differentially phosphorylating a number of the factors involved in the multifactor complex (eIF2α, eIF2β, eIF3c, and eIF5; Chapter 2). Of particular interest are modifications that affect the eIF3c N-terminal domain and the eIF5 C-terminal HEAT domain, both of which have been shown to play a central role in multifactor complex assembly in yeast (Hinnebusch et al., 2007). These critical binding domains appear to be conserved in plant initiation factors, and both domains in wheat initiation factors appear to be phosphorylated by CK2 isoforms on conserved residues (Chapter 3). The results presented here provide biochemical evidence that CK2 phosphorylation enhances the interaction of subunits

within the plant multifactor complex, and thus may have a regulatory role in the initiation of protein synthesis in plants.

## 4.2. METHODS

### 4.2.1. Materials

All chemicals were of high quality and obtained from Sigma, Fisher, VWR or as indicated. Ni-nitrilotriacetic acid (NTA) Superflow (Qiagen) was used according to manufacturer's instructions to purify 6XHis tagged substrates. The mMessage mMachine T7 Kit (Ambion) and Protein A IgG Plus Orientation Kit (Pierce) were also used according to manufacturer's instructions. The buffers used for chromatography were Buffer B (20 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and KCl as indicated, Buffer B-500 is Buffer B containing 500 mM KCl), Buffer C (50 mM HEPES-KOH pH 7.6, 600 mM KCl, 20 mM imidazole) and Buffer D (20 mM HEPES-KOH pH 7.6, 250 mM imidazole, and KCl as indicated).

### 4.2.2. Cloning, Expression, and Purification of Recombinant Initiation Factors and CK2

To obtain plant initiation factors for biochemical analysis, wheat initiation factors were cloned and expressed in *E. coli* as previously described (Chapter 2). Many of the factors in which purification was previously facilitated by a 6xHis-tag were cloned into new vetors for the expression of recombinant wild-type factors.

*eIF1.* The cDNA for wheat eIF1 was prepared *de novo* by the use of overlapping oligonucleotides designed by DNA_Works (http://helixweb.nih.gov/dnaworks/) based on

the protein sequence predicted by the TIGR gene indice TC233522 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat) and cloned into pET23b(+) using *NdeI/EcoRI* restriction sites. This synthetic construct was used to express and purify eIF1 as previously described (Mayberry et al., 2007).

*eIF2*. Wheat eIF2α (p42) was previously cloned into pET30a(+) for bacterial expression of His-eIF2α (Chang et al., 1999). To express wild-type eIF2α the coding region was PCR amplified from this construct and cloned into the *NcoI/BamHI* restriction sites of pET15b(+). Cell extract was obtained from 2 X 1L LB as previously described, and diluted with Buffer B to 50 mM KCl. The extract was then loaded onto a 3 ml phosphocellulose column equilibrated in Buffer B-50. After the supernatant was loaded, the column was washed with Buffer B-50 until the $A_{280}$ returned to baseline. Recombinant eIF2α was eluted from the column using a 4x gradient (12 ml) from 50 to 150 mM KCl in Buffer B. 50 ul aliquots of each fraction were taken and 10 ul of each aliquot was analyzed by SDS-PAGE to evaluate the purity of the recombinant eIF2α. The cleanest fractions (4ml) were pooled and dialyzed overnight at 4°C against Buffer B-100.

Wheat eIF2γ (p50) was cloned from expressed sequence tag (EST) 131 and 132. When sequenced it was determined that clone 131 was C-terminally truncated, while 132 contained an internal stop. In order to obtain a full length coding sequence for eIF2γ, EST 132 was PCR amplified and ligated into pET23b(+) using NdeI/NotI restriction sites. The internal stop codon was removed by replacing an internal *NcoI/NcoI* 687 bp fragment with the same fragment from EST 131. The newly produced eIF2γ-pET23b(+) clones were screened for orientation of the *NcoI/NcoI* fragment and the new construct

was verified by DNA sequencing. For bacterial expression, eIF2γ-pET23b(+) was used to transform Bl21(DE3) *E.coli*, and expressed using the procedure previously described. The cell extract was diluted with Buffer B to 80 mM KCl, and loaded onto a 3 ml phosphocellulose column equilibrated in Buffer B-80. After the supernatant was loaded, the column was washed with Buffer B-80 until the $A_{280}$ returned to baseline. Recombinant eIF2γ was eluted from the column using a 4x gradient (12 ml) from 80 to 200 mM KCl in Buffer B. 50 µl aliquots of each fraction were taken and 10 µl of each aliquot was analyzed by SDS-PAGE to evaluate the purity of the eluted eIF2γ. The cleanest fractions (4 ml) were pooled and dialyzed overnight at 4°C against Buffer B-100. Recombinant wheat eIF2β (p38) was prepared as previously described (Metz and Browning, 1997).

*eIF3c*. His-eIF3c-NTD was prepared as previously described (Chapter 3). To obtain recombinant wild-type eIF3c, the full-legnth cDNA for eIF3c was PCR amplified from expressed sequence tag *BJ257961* and cloned into pET23d(+) using *NcoI/SacI* restriction sites. This construct was transformed into Arctic Express DE3(RIL) *E. coli* cells (Stratagene). A 50 ml culture was inoculated with a single colony and grown overnight at 37°C. The overnight culture was used to inoculate 2 x 1 L of LB. Cells were grown at 25° C to an $A_{600}$ of 0.5, induced with 0.6 mM IPTG, and allowed to grow for 3 hr at 25°C. Cells were harvested by centrifugation as previously described and resuspended in 30 ml Buffer B-500 supplemented with 1 small Complete protease mini inhibitor tablet (Roche), 4.2 mg of soybean trypsin inhibitor (STI, Sigma), and 4.2 mg of phenylmethyl-sulfonyl fluoride (PMSF, Sigma) pre-dissolved in 0.5 ml of isopropanol.

The cells were disrupted by sonication as previously described and centrifuged at 184,048 x *g* for 30 min at 4$^{o}$C. Cell lysates were filtered using a 0.2 micron filter (Millipore) and diluted with Buffer B to lower the salt concentration to 100 mM KCl. The cell lysated was then loaded onto a 2 ml phosphocellulose column equilibrated in Buffer B-100. eIF3c was eluted from phosphocellulose with a 10X gradient (20 ml) from 100 to 500 mM KCl and 1 ml fractions were collected. Fractions were evaluated by SDS-PAGE and the peak eIF3c containing fractions were pooled, concentrated to approximately 1 ml using an Amicon Ultra-4 centrifugal filter (Millipore) and stored at -80$^{o}$C

*eIF5*. The cloning, expression, and purification of recombinant wheat His-eIF5 has been previously described (Mayberry et al., 2007). To evaluate the role of CK2 phosphorylation on eIF5, site-directed mutagenesis was performed to alter the previously identified phosphorylation sites S209, T240, and S451 to alanine or glutamate. The expression and purification of wheat His-eIF5 mutants has been described (Chapter 2). To express wild-type wheat eIF5 which did not possess a 6xHis-tag, the eIF5 coding region of His-eIF5-pET15b was cleaved with *Nde/BamHI* and cloned into pET-23b(+). This construct was transformed into BL21(DE3) *E. coli* and wild-type eIF5 was expressed as described above. The cell extract was diluted with Buffer B to 150 mM KCl, and loaded onto a 2 ml phosphocellulose column equilibrated in Buffer B-150. After the supernatant was loaded, the column was washed with Buffer B-150 until the $A_{280}$ returned to baseline. Recombinant wheat eIF5 was eluted from the column using a 5X gradient (10 ml) from 150 to 500 mM KCl in Buffer B, and 10 ul of each fraction was analyzed by SDS-PAGE to evaluate the purity of the eluted eIF5. Peak eIF5 fractions

were pooled and dialyzed in Buffer B-80. The sample was then loaded onto a 1 mL High Trap Q FF column equilibrated in Buffer B-80. Sample elution was performed by FPLC with a 10X gradient (10 ml) of 80 to 350 mM KCl in Buffer B and 10 ul of each fraction was analyzed by SDS-PAGE to evaluate the purity of the eluted eIF5. Fractions were stored at -80$^{o}$ C.

*CK2*. The cloning, expression, and purification of recombinant *A. thaliana* CK2 α and β subunits were previously performed with the aid of C-terminal 6xHis-tags (Chapters 1 & 2). Since Ni-NTA binding experiments were to be performed in this study, the removal of the 6xHis-tag was desirable. *A. thaliana* CK2α1 was PCR amplified from the previously used CK2α1-pET23d plasmid and cloned into pET23b using *NdeI/BamHI*. The new CK2α1-pET23b plasmid was transformed into BL21(DE3) *E. coli* and was expressed as described above. The cell extract was diluted with Buffer B to 300 mM KCl and loaded onto a 1 ml phosphocellulose column. CK2α1 was then eluted with a 10X gradient (10 ml) of Buffer B containing 0.3 to 1 M KCl. Fractions were analyzed by SDS-PAGE, and those containing the highest purity and concentration were pooled (5 ml). CK2α fractions were dialyzed overnight at 4$^{o}$C in Buffer B-100.

### 4.2.3. NMR Spectroscopy

Recombinant His-eIF5 and His-eIF5AAA were analyzed to evaluate protein folding using 1-dimensional (1D) NMR spectroscopy. NMR analysis was performed by Dr. David Hoffman at the University of Texas Department of Chemistry and Biochemistry. Sample preparation, spectra measurement and evaluation for 1D NMR spectroscopy were preformed as previously described (Monzingo et al., 2007).

### 4.2.4. Ni-NTA Pulldown Assay

Approximately 400 pmoles of C-terminally 6xHis-tagged eIF3c-NTD or N-terminally 6xHis-tagged-eIF5 was mixed with the indicated amounts and combinations of wheat eIF1, eIF2, eIF3c, and eIF5 in the presence of binding buffer (20 mM Hepes-KOH pH 7.6, 10% glycerol, 150 mM KCl, 0.1% Nonidet P-40, 20 mM imidazole) and Ni-NTA resin (Qiagen). A similar reaction was conducted in the absence of the 6xHis-tagged protein to control for any interaction between wheat initiation factors and the Ni-NTA matrix. The mixture was incubated for 15 min at room temperature with end-over-end mixing or for up to 12 hours at 4$^{o}$C. The beads were then extensively washed with binding buffer, and the bound proteins were eluted with 1xSDS containing 300 mM imidazole and separated by SDS-PAGE. Assays were then either visualized by Coomassie staining or western analysis.

### 4.2.5. Development of Antibodies for Wheat Initiation Factors

Custom antiserum was raised to recombinant wheat initiation factors (eIF1, eIF2α, His-eIF3c, and His-eIF5) using two New Zealand white rabbits and a 77-day immunization and bleed schedule (Sigma-Genosys). The initial immunization was given in Complete Freund's Adjuvant and all subsequent immunizations in Incomplete Freund's Adjuvant. Antisera were used in the indicated dilutions and incubations.

### 4.2.6. Western Analysis

Following separation by SDS-PAGE, proteins were blotted to PVDF and blocked with HNAT (10 mM Hepes-KOH pH 7.6, 150 mM NaCl, 0.2% BSA, 0.2% Tween 20)

containing 5% dry milk. Blots were then incubated with primary antibodies as indicated followed by extensive washes with HNAT/milk soluntion. The second antibody incubation contained a 1/20,000 dilution in HNAT/milk of goat-anti-rabbit-HRP secondary antibody for 1 hr at room temperature. Following extensive washes, antibody reactive bands were then visualized by chemiluminesence (SuperSignal, Pierce) and exposure to film. To analyze blots with multiple antibodies, the membrane was treated with stripping buffer (5% SDS, 20 mM β-mercapatoethanol, and 125 mM Tris-HCl pH 6.8) for 30 min at 65$^{o}$C, blocked with HNAT containing 5% milk and incubated with a different primary antibody. PVDF membranes were stripped 3-4 times with no apparent loss of bound recombinant factors.

**4.2.7. Preparation of eIF5-depleted Wheat Germ Extracts**

Wheat germ S30 extracts were prepared as previously described (Lax et al., 1986), and depleted of eIF5 using Protein A beads (Pierce) crosslinked to polyclonal rabbit antibodies raised against recombinant wheat eIF5 (Rαwt5, Sigma). A 2 ml Protein A anti-wheat eIF5 column was prepared using the Protein A IgG Plus Orientation Kit (Pierce) according to the manufacturer's instructions. Briefly, IgG was precipitated from 12 ml of rabbit antisera by the addition of 50% $(NH_4)_2SO_4$. The precipitated IgG protein was resuspended in 2 ml of phosphate buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 8.0) and dialyzed for 3 hr at 4$^{o}$C against 1 L of phosphate buffer. The sample was clarified by centrifugation at 12,000 x *g* prior to incubation with Protein A beads. Protein A beads were equilibrated in Antibody Binding/Wash Buffer (Pierce), allowed to incubate with the IgG fraction for 30 min, and washed with 5 ml of Antibody Binding/Wash buffer. The bound IgG was crosslinked to the protein with disuccinimidyl

suberate (DSS) for 1 hr, and washed with phosphate buffer. The remaining un-reacted DSS groups were then blocked with Blocking Buffer (Pierce), and the column was washed several times with IgG Elution Buffer (Pierce) followed by Antibody Binding/Wash Buffer. The column was then equilibrated with 20 mL of Buffer A (20 mM Hepes-KOH pH 7.6, 5 mM $Mg(OAc)_2$, 120 mM KOAc, 6 mM BME (2-mercaptoethanol), 10% glycerol) . Wheat germ S30 extract (15 ml) was passed over the column and 1 ml fractions were collected. The S30 flow-through fractions were analyzed by western and the eIF5-depleted fractions were stored in 100 µl aliquots at –80$^{o}$C.

### 4.2.8. *In vitro* Transcription of HSP21 mRNA

Capped plant heat shock protein 21 (HSP21) mRNA was prepared as previously described (Browning and Mayberry, 2006). Briefly, mRNA was transcribed from linearized plasmid using mMessage mMachine T7 kit (Ambion) according to manufacturer's instructions. Transcription reactions (0.5-1.0 mL reactions) were purified using a 20 ml sterile Sephadex G100 column equilibrated in 20 mM Hepes-KOH pH 7.5, 0.1 M EDTA, 150 mM KCl. Peak fractions were pooled, phenol extracted, ethanol precipitated, and resuspended in sterile water.

### 4.2.9. eIF5-dependent *in vitro* Translation Assay

Recombinant eIF5 was assayed for its ability to stimulate translation in the wheat germ S30 assay system (Timmer et al., 1993; McInerney et al., 2006) using eIF5-depleted wheat germ extracts. Briefly, 50 µl reactions containing 5 pmol HSP21 mRNA

template, recombinant eIF5 (as indicated), 4 pmol recombinant wheat eIF4F (Mayberry et al., 2007), 24 mM Hepes-KOH pH 7.6, 2.9 mM $Mg(OAc)_2$, 100 mM KOAc, 30 mM KCl, 2.4 mM DTT, 0.1 mM spermine, 1 mM ATP, 0.2 mM GTP, 34 uM [$^{14}$C]leucine, 50 μM of 19 amino acids (minus leucine), 7.8 μM creatine phosphate, 3 μg creatine kinase, 0.75 $A_{260}$ units of yeast tRNA, and 20 μl of eIF5-depleted wheat germ extract. Reactions were incubated for 30 min at 27$^{o}$C and the amount of [$^{14}$C]leucine incorporated into polypeptide was determined as previously described (Lax et al., 1986; McInerney et al., 2006).

## 4.3. RESULTS AND DISCUSSION

### 4.3.1. Interaction of Wheat Initiation Factors with eIF3c-NTD *in vitro*

The N-terminal domain of wheat eIF3c was found to directly interact *in vitro* with eIF1, eIF2, and eIF5 (Figure 4.1, lanes 1, 3, & 5 respectively) in formation of the multifactor complex using Ni-NTA pulldowns of His-eIF3c-NTD. In addition, the ability of His-eIF3c-NTD to bind to individual factors is enhanced in the presence of eIF1, eIF2, and eIF5 (Figure 4.1, compare lanes 1, 3, &, 5 to lane 7). This cooperative binding could be the result of additional bridging interactions between various subunits, or may be the result of structural modifications that occur in the eIF3c N-terminus upon interaction with factors. It has been previously observed that the physical association of yeast eIF5 with the eIF2β N-terminus enhances the eIF3/eIF5 interactions, although no direct interaction could be demonstrated between eIF2 and eIF3 (Singh et al., 2004). The interaction of eIF1 with the eIF3c N-terminus has been previously reported with yeast and mammalian

factors (Asano et al., 1998; Fletcher et al., 1999); however, it was not known if similar interactions occurred with plant factors. The binding of recombinant wheat eIF1 to His-eIF3c-NTD suggests that a similar interaction takes place in plants. The interaction of recombinant wheat eIF1 with eIF3c-NTD occurs weakly under the *in vitro* binding conditions used in Figure 4.1. This assay was conducted at 150 mM KCl, and at lower salt concentrations (~50 mM KCl) the eIF3c/eIF1 interaction is more obvious (data not shown). In addition to binding to eIF1, the N-terminal segment of yeast eIF3 (NIP1) is known to simultaneously interact with eIF5 (Asano et al., 2000). The findings presented here indicate that eIF1, eIF2, and eIF5 are capable of binding to the His-eIF3c-NTD *in vitro*.

In addition to binding recombinant wheat eIF1 and eIF5, His-eIF3c-NTD unexpectedly bound to native wheat eIF2 in Ni-NTA pulldown experiments. The direct interaction of eIF3c with eIF2 has not been previously reported in any system, and further experiments are underway to determine which subunit of wheat eIF2 mediates this interaction. A preliminary examination suggests a strong direct interaction between recombinant eIF2β and the eIF3c-NTD (data not shown). It is possible that this represents a plant-specific multifactor complex subunit interaction. An indirect interaction of these subunits has been observed in yeast, as eIF3a (TIF32), eIF1, and eIF5 all serve as intermediaries, binding simultaneously to both eIF3c (NIP1) and the β-subunit of eIF2 (Valasek et al., 2002; Reibarkh et al., 2008). Further analysis with individual recombinant wheat eIF2 subunits will provide information on this interaction. These findings represent the first biochemical evidence of multifactor complex formation with plant initiation factors.

The N-terminal domain of wheat eIF3c is highly phosphorylated by CK2 *in vitro* (Chapter 3), and the presence of CK2 significantly increased the interaction of His-eIF3c-NTD with eIF1, eIF2, and eIF5 in Ni-NTA pulldowns (Figure 4.1, compare lanes 1, 3, & 5 with 2, 4, & 6, respectively). When eIFs 1, 2, and 5 are all three present, the binding of individual subunits to His-eIF3c-NTD is increased as previously mentioned; however, the presence of CK2 still provides a further enhancement in the binding of eIFs 1, 2, and 5 to the His-eIF3c-NTD (Figure 4.1, compare lanes 7 and 8). CK2 phosphorylates eIF2α, eIF2β, and eIF5 *in vitro* (Chapter 2), thus the increased binding in the presence of CK2 may not be solely attributed to the phosphorylation of eIF3c, but may represent multiple phosphorylation events.

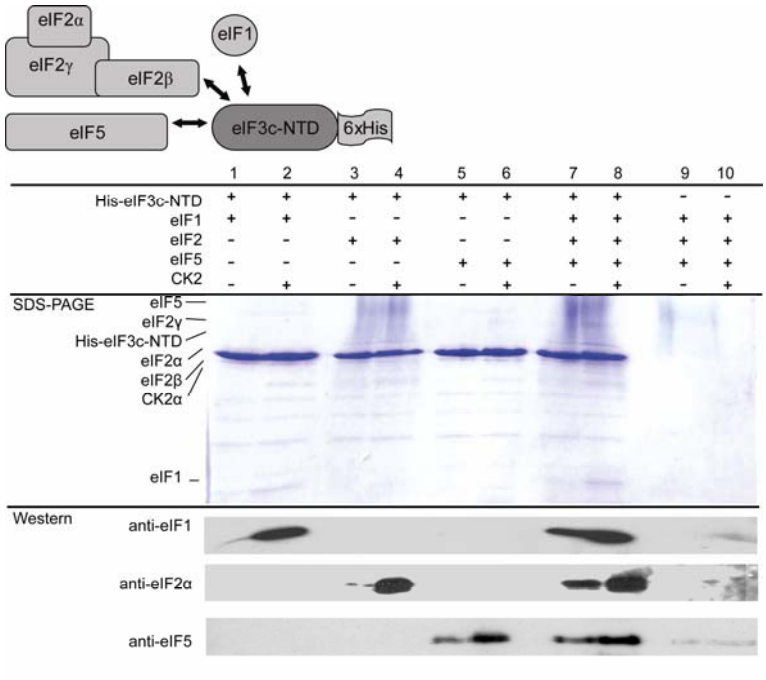



**Figure 4.1. CK2 enhances interaction of MFC components with eIF3c NTD *in vitro***

6xHis-eIF3c-NTD (400 pmoles) was mixed with approximately 400 pmoles of the following initiation factors as indicated: recombinant wheat eIF1, native wheat eIF2 (Lax et al., 1986), and/or recombinant wheat eIF5. Each 400 µl reaction contained 20 mM Hepes-KOH pH 7.6, 10% glycerol, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1% Nonidet P-40, 20 mM imidazole and 10 µl Ni-NTA resin (Qiagen). 10 pmoles of recombinant *A. thaliana* CK2α1 was used to phosphorylate initiation factors. Control reactions (lanes 9 & 10) were conducted in the absence of the 6xHis-tagged protein to evaluate the interaction between wheat initiation factors/CK2α and the Ni-NTA beads. The mixture was incubated for 30 min at room temperature with gentle mixing. The beads were then extensively washed with binding buffer, and the bound proteins were extracted with 40 µl of 1XSDS loading buffer containing 300 mM imidazole. The eluted proteins were separated by SDS-PAGE, and visualized by Coomassie Blue staining or transferred to PVDF and analyzed with rabbit antisera to recombinant wheat eIF2α, eIF5, and eIF1. The blot was incubated for 3 hr at room temperature in a 1/1000 dilution of rabbit antisera to recombinant wheat eIF2α. The blot was washed extensively in HNAT/milk, followed by a 1 hr incubation at room temperature in a 1/20,000 dilution of goat-anti-rabbit-HRP secondary antibody. The antibody reactive bands were visualized by chemiluminesence (SuperSignal, Pierce) and exposed to film. The blot was then stripped with 5% SDS, 20 mM β-mercapatoethanol, and 125 mM Tris-HCl pH 6.8 for 30 min at 65$^{o}$C and the western blotting cycle was repeated using a 1/5000 dilution of rabbit antisera to recombinant eIF5, and then a 1/5000 dilution of rabbit antisera to recombinant eIF1. For an enlarged image see Appendix.

A growing body of evidence suggests that eIF3, a highly complex initiation factor consisting of 8-13 subunits, may function as the central hub in the coordination of translation initiation (Hinnebusch, 2006). The interaction of residues in the N-terminal domain of yeast eIF3c (NIP1) with eIF1 and eIF5 has been shown to play an important role in AUG selection; these interactions appear to link the function of eIF1 in recognizing proper codon-anticodon base pairing with both the GAP activity of eIF5 and the release of the Pi generated by eIF2-GTP hydrolysis (Valasek et al., 2004). In this study, a similar binding event has been demonstrated with recombinant wheat eIF3c residues, and this binding was enhanced by the presence of CK2. It is likely that the phosphorylation of the eIF3c-NTD by CK2 enhances its interaction with positively charged binding domains in its multifactor complex partners, as basic domains containing high levels of lysine and arginine residues in yeast eIF1 (Reibarkh et al., 2008) and eIF5 (Yamamoto et al., 2005) play a critical role in their association with eIF3c. For the scope of this study, mutagenesis of CK2 phosphorylation sites in eIF3c was not practical, as 12 *in vitro* phosphorylation sites were previously identified (Chapter 3). However, the 3 sites in eIF5 presented a viable mutagenesis target to test the role of CK2 phosphorylation.

### 4.3.2. Interaction of Wheat Initiation Factors with eIF5 *in vitro*

Recombinant wheat His-eIF5 directly interacts with wheat recombinant eIF1, native eIF2, and eIF3c *in vitro* (Figure 4.2, lanes 1, 3, & 5). The interaction of recombinant wheat eIF1 and full-length eIF3c with His-eIF5 occurs modestly under the

*in vitro* binding conditions used in this study; however, the amount of these factors pulled down by His-eIF5 is enhanced in the presence of all three factors involved in multifactor complex formation (native wheat eIF2, recombinant wheat eIF1, and recombinant full-length wheat eIF3c; Figure 4.2 compare lanes 1, 3, & 5 with 2, 4, & 6, respectively). This enhanced interaction is likely due to the cooperative binding of factors, as interactions have also been demonstrated between eIF3c-NTD and both eIF1 and eIF2. Assays conducted in the presence of native wheat eIF3 were inconclusive, as the eIF3 complex bound to the Ni-NTA matrix in the absence of any His-tagged proteins. This interaction of eIF3 with Ni-NTA was detected even when reactions were conducted in the presence of 50 mM imidazole. The cause of this non-specific interaction is unknown, as a sequence analysis of eIF3 subunits revealed no more than 2 consecutive histidine residues.

In Ni-NTA binding assays, the interaction of native wheat eIF2 or recombinant eIF2β with His-eIF5 could be easily detected by Coomassie staining (Figure 4.3). The interaction of the eIF5 C-terminus with the N-terminal domain of eIF2β has been previously demonstrated in yeast (Asano et al., 1999). AA-boxes (acidic/aromatic) in the C-terminal HEAT domain of yeast eIF5 mediate interaction with eIF2 via the lysine rich K-boxes of eIF2β (Asano et al., 1999). In Figure 4.4, increasing salt concentration inhibited the interaction of wheat eIF5 with eIF2β, suggesting a similar ionic interaction occurs in plants.

In the presence of CK2, the binding of eIF1 and eIF3c to His-eIF5 is enhanced in pulldown assays (Figure 4.2, compare lanes 1 & 5 with lanes 2 & 6); however, a similar increase in the interaction of eIF2 with eIF5 has not been clearly demonstrated *in vitro*

(compare lanes 3 & 4). Studies in mammalian cells suggest that the eIF2/eIF5 interaction may be dependent on CK2 phosphorylation of eIF5 (Homma et al., 2005). In the wheat system, *in vitro* Ni-NTA binding assays suggest that CK2 has no effect on the direct interaction of wheat eIF2 or recombinant eIF2β with His-eIF5 (Figure 4.3). It was initially suspected that this was the result of *in vitro* binding conditions being highly favorable, thus any increase in interaction that was mediated by CK2 was negligible. However, even in the presence of higher salt concentrations (150 mM KCl) and lower protein concentrations (~20 nM), no clear effect of CK2 phosphorylation could be demonstrated on eIF2/eIF5 binding. In this study, CK2 phosphorylation of wheat eIF5 did not directly affect its *in vitro* interaction with eIF2; however, it cannot be ruled out that this interaction is modified by CK2 phosphorylation *in vivo*. The increased interaction of full-length wheat eIF3c with His-eIF5 in the presence of CK2 supports similar results with His-eIF3c-NTD and wild-type eIF5. In addition, this study provides the first evidence that CK2 phosphorylation of wheat eIF5 influences its interaction with eIF1.

The eIF1/eIF5 interaction reported here has been previously observed in yeast (Reibarkh et al., 2008); however, the interaction of human eIF1 with recombinant human eIF5 could not be demonstrated (Fletcher et al., 1999). It is likely that the interaction of eIF1 with eIF3c stabilizes the eIF1/eIF5 interaction, as the His-eIF3c-NTD was found to bind both eIF1 and eIF5. This could also be the case for wheat eIF2; however, the direct interaction of wheat eIF2 and eIF1 has not yet been demonstrated with plant initiation factors. Yeast eIF1 has been shown to interact with all three factors involved in the

multifactor complex (Reibarkh et al., 2008), thus it is conceivable that eIF2 may enhance the eIF1/eIF5 interaction through a similar bridging interaction.

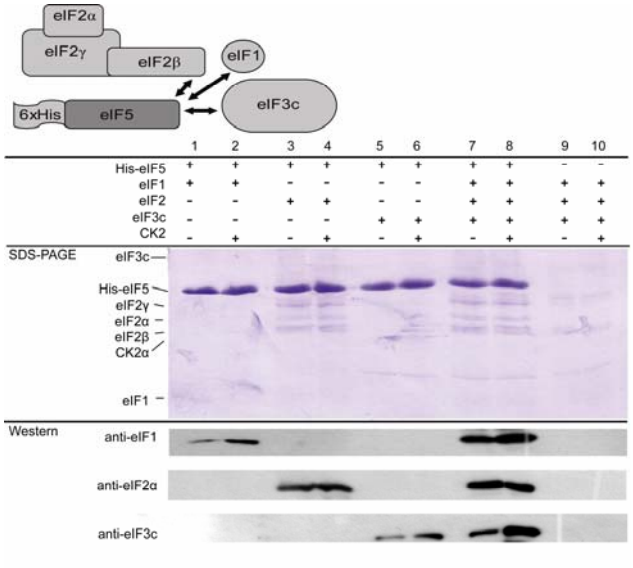



**Figure 4.2. CK2 enhances interaction of MFC components with His-eIF5 *in vitro***

6xHis-eIF5 (250 pmoles) was mixed with approximately 250 pmoles of the following initiation factors as indicated: recombinant wheat eIF1, native wheat eIF2 (Lax et al., 1986), and/or recombinant wheat eIF3c. Each 500 µl reaction contained 20 mM Hepes-KOH pH 7.6, 10% glycerol, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1% Nonidet P-40, 20 mM imidazole and 10 µl Ni-NTA resin (Qiagen). 10 pmoles of recombinant *A. thaliana* CK2α1 was used to phosphorylate initiation factors. Control reactions (lanes 9 & 10) were conducted in the absence of the 6xHis-tagged protein to evaluate the interaction between wheat initiation factors/CK2α and the Ni-NTA beads. The mixture was incubated for 30 min at room temperature with gentle mixing, followed by overnight incubation at 4$^{o}$C. The beads were then extensively washed with binding buffer, and the bound proteins were extracted with 30 µl of 1XSDS loading buffer containing 300 mM imidazole. The elution was separated by SDS-PAGE, and visualized by Coomassie Blue staining or transferred to PVDF and analyzed with rabbit antisera to recombinant wheat eIF2α, eIF3c, and eIF1. The blot was incubated for 3 hr at room temperature in a 1/1000 dilution of rabbit antisera to recombinant wheat eIF2α. The blot was washed extensively in HNAT/milk, followed by a 1 hr incubation at room temperature in a 1/20,000 dilution of goat-anti-rabbit-HRP secondary antibody. The antibody reactive bands were visualized by chemiluminesence (SuperSignal, Pierce) and exposed to film. The blot was then stripped with 5% SDS, 20 mM β-mercapatoethanol, and 125 mM Tris-HCl pH 6.8 for 30 min at 65oC and the western blotting cycle was repeated using a 1/5000 dilution of rabbit antisera to recombinant eIF3c, followed by a 1/5000 dilution of rabbit antisera to recombinant eIF1. For an enlarged image see Appendix Figure 4.2.

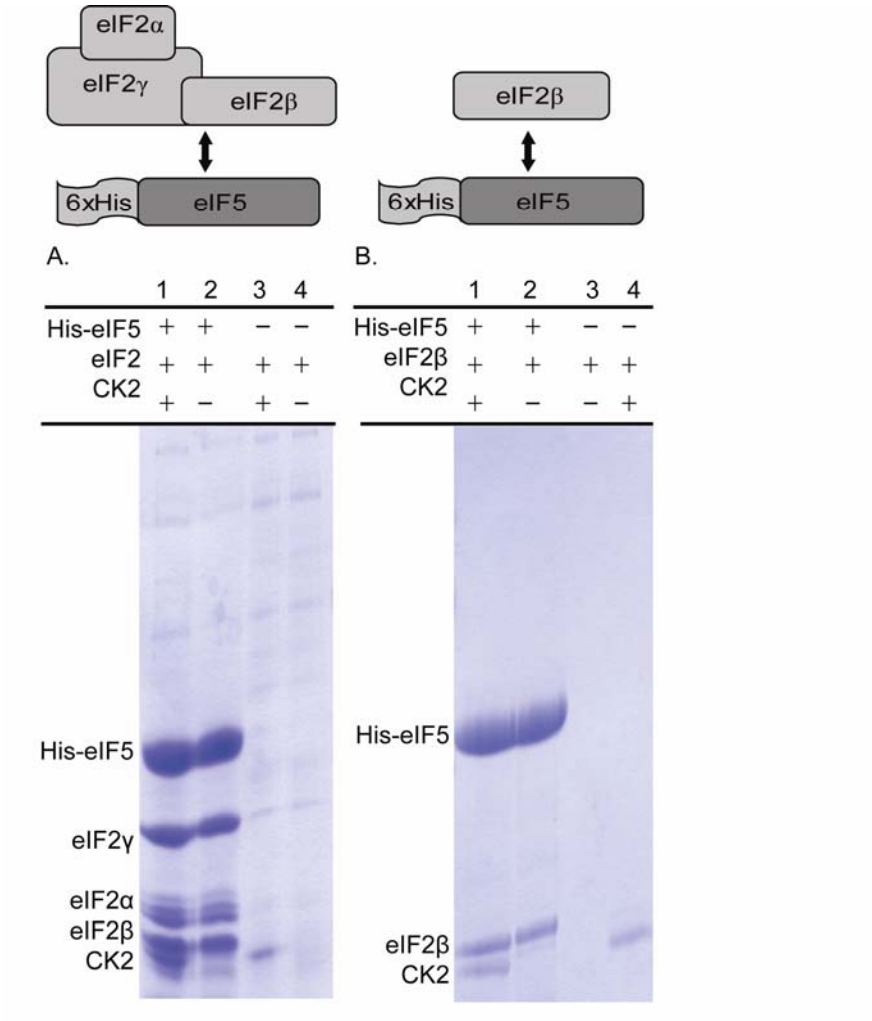



**Figure 4.3. Wheat eIF2 and recombinant eIF2β bind His-eIF5 *in vitro***

6xHis-eIF5 (400 pmoles) was mixed with approximately 400 pmoles of native wheat eIF2 (Lax et al., 1986) or recombinant wheat eIF2β. Each 500 µl reaction contained 20 mM Hepes-KOH pH 7.6, 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1% Nonidet P-40, 20 mM imidazole and 10 µl Ni-NTA resin (Qiagen). 10 pmoles of recombinant *A. thaliana* CK2α1 was used to phosphorylate initiation factors. Control reactions (lanes 9 & 10) were conducted in the absence of the 6xHis-tagged protein to evaluate the interaction between wheat initiation factors and the Ni-NTA beads in the presence and absence of His-CK2α. The mixture was incubated for 30 min at room temperature with gentle mixing. The beads were then extensively washed with binding buffer, and the bound proteins were extracted with 40 µl of 300 mM imidazole elution buffer. The elution was separated by SDS-PAGE and stained with Coomassie.

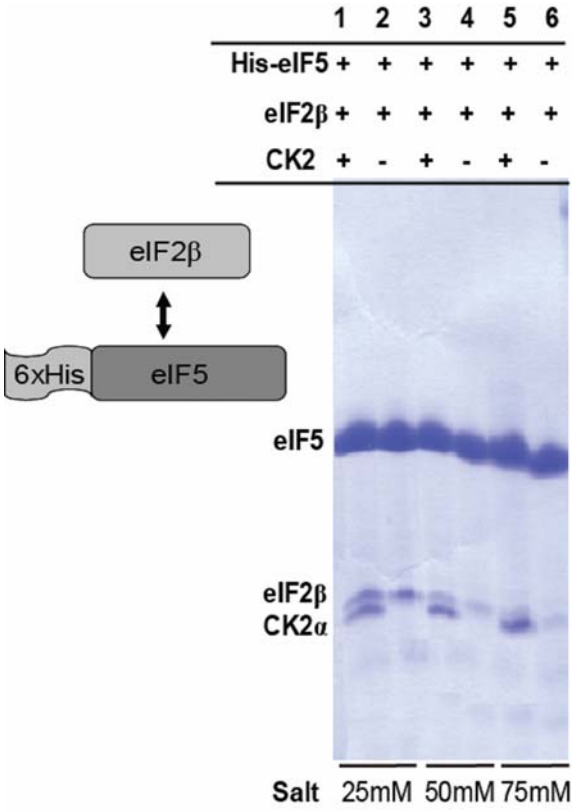



**Figure 4.4. Increasing salt concentrations inhibits the interaction of eIF2β and His-eIF5 *in vitro***

6xHis-eIF5 (400 pmoles) was mixed with approximately 400 pmoles recombinant wheat eIF2β in the presence of varying levels of salt. Each 500 µl reaction contained 20 mM Hepes-KOH pH 7.6, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1% Nonidet P-40, 20 mM imidazole and 10 µl Ni-NTA resin (Qiagen). 10 pmoles of recombinant *A. thaliana* CK2α1 was used to phosphorylate initiation factors. Control reactions (lanes 9 & 10) were conducted in the absence of the 6xHis-tagged protein to evaluate the interaction between wheat initiation factors and the Ni-NTA beads in the presence and absence of CK2α.

Mutation of the three eIF5 CK2 phosphorylation sites to alanine (His-eIF5AAA) prevents the CK2 mediated increase in eIF1 binding (Figure 4.5, compare lanes 1 and 3). This suggests that the increase in eIF1/eIF5 interaction is mediated by phosphorylation of eIF5. Both recombinant wheat His-eIF5 and His-eIF5AAA appear to be folded in 1D NMR spectra (Figure 4.6), suggesting that the change in binding is not due to misfolding of the protein. It was recently reported that a basic/hydrophobic surface of yeast eIF1 participates in binding to the eIF5 C-terminus, and mutations that alter basic residues in this domain of eIF1 were lethal (Reibarkh et al., 2008). It is likely that phosphorylation of wheat eIF5 enhances interactions with positively charged domains in wheat eIF1.

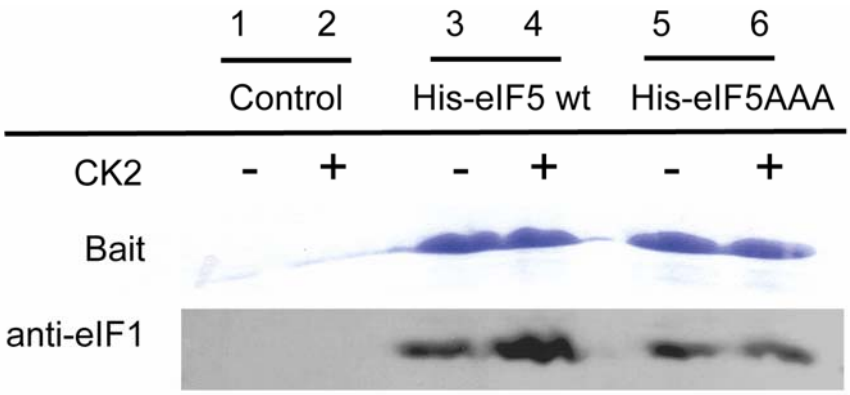



**Figure 4.5. Increased *in vitro* interaction with eIF1 upon phosphorylation of eIF5.**

Phosphorylation of recombinant wheat His-eIF5 by CK2 enhances its interaction with eIF1. Alanine mutations at 3 CK2 phosphorylation sites (S209A, T240A, S451A; His-eIF5AAA) prevent the CK2 mediated increase in eIF1 binding to eIF5. N-terminally 6xHis-tagged eIF5 (400 pmoles) or eIF5AAA was mixed with approximately 400 pmoles of recombinant wheat eIF1 in a 300 µl reaction containing 20 mM Hepes-KOH pH 7.6, 10% glycerol, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.1% Nonidet P-40, 20 mM imidazole and Ni-NTA resin (Qiagen). 10 pmoles of recombinant *A. thaliana* CK2α1 was used to phosphorylate initiation factors. Control reactions were conducted in the absence of the 6xHis-tagged protein to evaluate the interaction between wheat eIF1 and 10 µl Ni-NTA matrix. The elution was visualized by Coomassie Blue staining, or transferred to PVDF and analyzed with a 1/5000 dilution of rabbit antisera to recombinant wheat eIF1. The blot was washed extensively in HNAT and incubated 1 hr at room temperature in a 1/20,000 dilution of goat-anti-rabbit-HRP secondary antibody. The antibody reactive bands were visualized by chemiluminesence (SuperSignal, Pierce) and exposed to film.

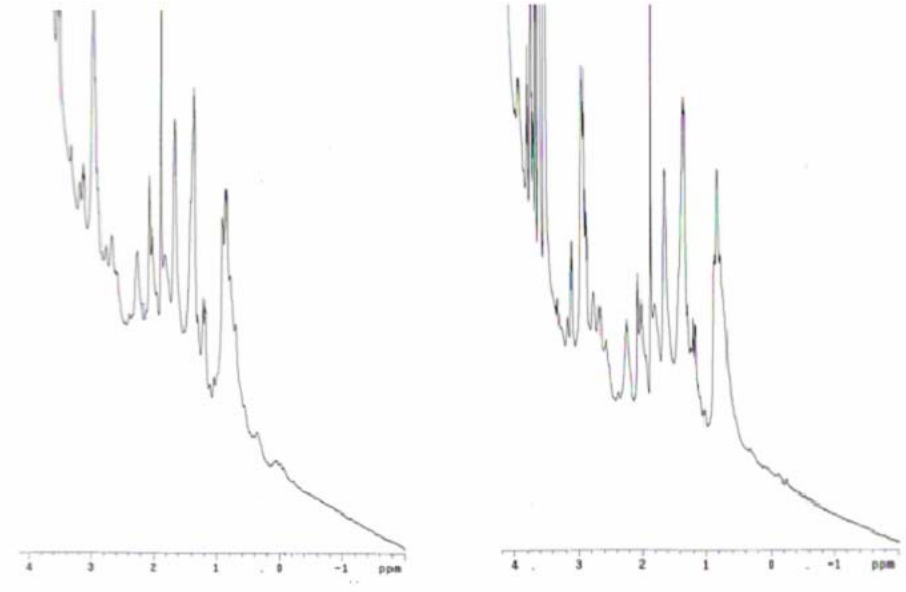



**Figure 4.6. 1D NMR analysis of recombinant wheat His-eIF5 and His-eIF5AAA**

One-dimensional NMR analysis was performed on recombinant wheat His-eIF5 (left) and the mutant His-eIF5AAA (right) to evaluate protein folding. Unfolded protein generally does not show any peaks below about 0.8 ppm. Peaks near 0 ppm are typical of methyl groups in folded protein. Based upon this analysis, both recombinant wheat His-eIF5 and His-eIF5AAA appear to be folded. NMR analysis was performed by Dr. David Hoffman at the University of Texas Department of Chemistry and Biochemistry.

### 4.3.3. Effect of eIF5 Mutagenesis on *in vitro* Translation

To evaluate the role CK2 phosphorylation plays on the events of translation initiation, site-directed mutagenesis was performed on recombinant wheat eIF5. Mutation of these three wheat eIF5 CK2 phosphorylation sites to alanine, as in eIF5AAA, prevents phosphorylation by CK2 (Chapter 3). The recombinant eIF5 and eIF5AAA preparations were analyzed by both Bradford and SDS-PAGE to ensure equivalent levels of protein were tested during *in vitro* analysis. Wheat germ S30 extracts were depleted of native eIF5 by an anti-eIF5 affinity column, and the ability of recombinant eIF5 to reactivate translational activity was evaluated by the incorporation of [$C^{14}$]leucine into polypeptide. The ability of recombinant wheat eIF5AAA to stimulate *in vitro* translation in eIF5-depleted wheat germ extracts is reduced by up to 50% compared to wild-type eIF5 (Figure 4.7). Similar results were obtained when assays were conducted in the presence of the phosphatase inhibitor calyculin A (data not shown). A similar finding has been reported *in vivo* with mammalian cells, where the expression of mutants that prevented CK2 phosphorylation at S389 and S390 disrupted synchronous cell cycle progression and significantly reduced growth rates (Homma et al., 2005). These findings suggest that the phosphorylation of wheat eIF5 by CK2 may play an important role in mediating this initiation factor's activity.

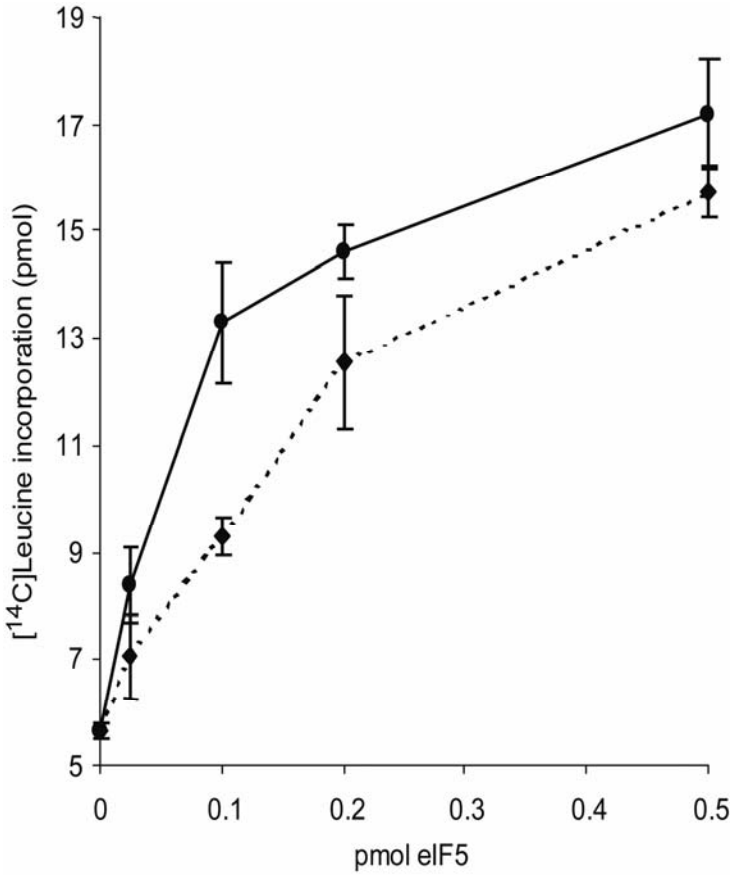



**Figure 4.7. eIF5-dependant translation assay**

The ability of recombinant wheat eIF5 to stimulate translation in eIF5-depleted wheat germ S30 extracts was analyzed. Each 50 µl reaction contained 20 µl of eIF5 depleted wheat germ extract, 5 pmol HSP21 mRNA template, and recombinant wheat eIF5 (—●—) or eIF5AAA (---■---) as indicated. The eIF5AAA mutant has all three CK2 phosphorylation sites mutated to alanine. Reactions were incubated for 30 min at 27°C and the amount of [$^{14}$C]leucine incorporated into polypeptide was determined as previously described (Lax et al., 1986; McInerney et al., 2006).

## 4.4. CONCLUSIONS

The results presented here suggest that plant initiation factors are capable of forming multifactor complexes similar to those previously reported in yeast (Asano et al., 2000). A series of *in vitro* interactions were observed between recombinant wheat eIF1, eIF3c, eIF5 and native wheat eIF2 (summarized in Figure 4.8). Within the context of the eIF3 complex, eIF3c may function differently, as native wheat eIF3 contains 12 additional subunits. Unfortunately, native wheat eIF3 non-specifically interacts with the Ni-NTA matrix used in pulldown assays. Using an alternative tag (FLAG), it will likely be possible to gain further insight into the interaction of initiation factors with native wheat eIF3. In preliminary pulldown assays, using polyclonal antibodies raised against recombinant wheat eIF3c, the interaction of wheat eIF1, eIF2, and eIF5 with native eIF3 has been observed (data not shown); however the effect of CK2 on this interaction is not known.

In earlier chapters of this work, the differential phosphorylation of wheat eIF2, eIF3 and eIF5 was demonstrated using recombinant CK2 holoenzymes (Chapter 2). When these phosphorylation sites were identified, it became obvious that a number of the critical multifactor complex interaction domains were the targets of plant CK2 phosphorylation. This finding gave rise to the hypothesis that the phosphorylation of wheat initiation factors by CK2 could play a role in regulating multifactor complex formation. These results demonstrate that the phosphorylation of initiation factors by CK2 enhances the interaction of multifactor complex components *in vitro*. Site-directed mutagenesis of eIF5 to remove CK2 phosphorylation sites, not only prevented the CK2

mediated increase in interaction with eIF1, but also resulted in reduced stimulation of translation initiation *in vitro*. These findings suggest a potential role for CK2 in regulating plant translation.

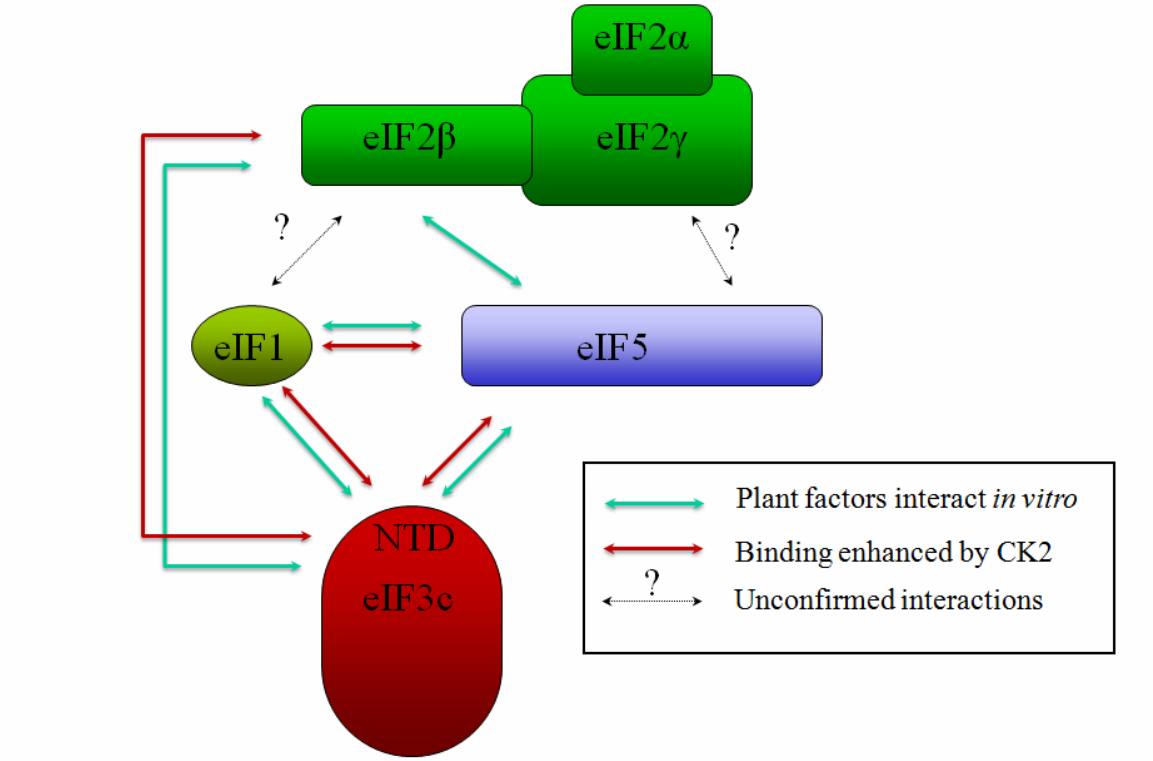



**Figure 4.8. Schematic illustration of wheat initiation factor *in vitro* interactions**

# Chapter 5: Conclusions and Future Work

## 5.1. CONCLUSIONS

The results presented in this dissertation suggest a potential role for CK2 in the regulation of plant translation initiation. Wheat extracts contain an active form of CK2, which is capable of phosphorylating a number of translation initiation factors, 60S ribosomes, as well as other substrates such as HD2β. We have successfully expressed and purified two recombinant CK2 catalytic subunits, and four recombinant CK2 regulatory subunits from *Arabidopsis thaliana*, which have enzymatic activity similar to the native wheat CK2. This work also demonstrates that holoenzyme formation of catalytic CK2α subunits with various regulatory CK2β subunits alters the ability of recombinant *A. thaliana* CK2α subunits to phosphorylate eIF2α, eIF2β, eIF3c, eIF4B, and eIF5 *in vitro*. Mammalian CK2 is typically viewed as being constitutively active; however, the regulated expression of multiple β-subunits in plants presents a novel mechanism for regulating kinase activity or substrate specificity. In order to evaluate the potential effects of CK2 phosphorylation on plant translation initiation, it was necessary to identify the residues that were modified by the kinase. Our analysis of initiation factor phosphopeptides produced by *in vitro* phosphorylation identified 20 CK2 phosphorylation sites in eIF2α, eIF2β, eIF3c, eIF4B, and eIF5. The purification of native wheat germ eIF5 revealed that it was phosphorylated at the S451, suggesting the *in vivo* relevance of CK2 sites that were identified *in vitro*.

The identification of wheat initiation factor substrates was particularly interesting since three of the four factors involved in forming the multifactor complex, which consists of eIF1/eIF3/eIF5/eIF2/GTP/Met-tRNA$_i^{Met}$, appear to be targets of CK2 phosphorylation. These complexes have been extensively studied in yeast; however, it was not known if a similar complex existed in plants. The results presented here suggest that plant initiation factors are capable of forming multifactor complexes similar to those previously reported in yeast. Phosphorylation site identification by mass spectrometry revealed that a number of CK2 phosphorylation sites were located in the N- and C-termini of these initiation factors, which participate in interactions within the yeast multifactor complex. This led to the hypothesis that CK2 regulates multifactor complex assembly, and we show that CK2 appears to enhance the interaction of wheat multifactor complex components *in vitro*. Site-directed mutagenesis of eIF5 to remove CK2 phosphorylation sites, not only prevented the CK2 mediated increase in interaction with eIF1, but also resulted in a reduced ability of recombinant eIF5 to stimulate translation initiation *in vitro*. Taken together, these findings suggest that CK2 may regulate plant translation initiation by mediating the assembly of functional initiation factor complexes.

## 5.2. FUTURE DIRECTIONS

### 5.2.1 Evaluation of CK2 Phosphorylation *in planta*

Wheat germ has proven itself as an invaluable resource for the biochemical study of plant translaiton. Unfortunately, with the benefits provided by this widely available resource comes the disadvantage of a quiescent system and an incomplete genome. This

study represents a starting point for evaluating the role CK2 phosphorylation plays in regulating plant translation initiation, and some direct *in vivo* evidence of phosphorylation has been presented to support the findings. In addition, a number of key tools have been developed during this study, including recombinant plant initiation factors, CK2 phosphorylation site mutants (eIF5, eIF2α), and antibodies for eIF1, eIF5, eIF3c, CK2α, and CK2β subunits. While these studies have provided important insight into the phosphorylation of initiation factors by CK2, further studies need to be performed in *A. thaliana* or with plant tissue culture.

While protein phosphorylation is known to be a key regulator of biological function in eukaryotes, relatively little is known about phosphorylation pathways in plants. Large-scale phosphoproteonomic analyses *in planta* are only recently gaining momentum, as technologies developed with yeast and mammals are being successfully adapted to plants. During the writing of this manuscript, the initial public release of the Arabidopsis Protein Phosphorylation Site (*PhosPhAt* 2.1*)* database provided *in vivo* evidence of eIF3b, eIF3c, eIF4B, and eIF4A phosphorylation (Heazlewood et al., 2008). However, a comprehensive analysis of plant initiation factor post-translational modifications is still lacking. Dr. Steven Clouse at North Carolina State University is currently conducting an *in vivo* phosphoproteomic analysis of *A. thaliana* eIFs to specifically determine the role of brassinosteroid responsive kinases in regulating plant growth and development. It is our hope, that by collaborating with Dr. Clouse, more extensive *in vivo* evidence of initiation factor phosphorylation by CK2 can be obtained. It is important to not only identify phosphorylation in metabolically active systems, but to

gain insight into how the phosphorylation patterns of translation initiation factors are affected by changes in cellular physiology and in response to stress.

Future studies must also investigate the role of CK2 *in vivo* using transgenic plants. While pursuing this research, clones were generated to induce the overexpression of *At*-CK2α and *At*-CK2β subunits by agroinfiltration. Initial attempts at generating transformants were unsuccessful; however, these clones represent a valuable tool for the future evaluation. It has been previously observed that overexpression of CK2β3 in *A. thaliana* resulted in 2-fold increase in CK2 activity, and plants exhibited shorter periods of rhythmic expression of circadian clock-associated 1 (CCA1) and the late elongated hypocotyl (LHY) (Sugano et al., 1999). As a result, the photoperiodic flowering response was diminished in transgenic lines and plants flowered earlier on both long-day and short-day photoperiods (Sugano et al., 1999). No other visible aspects of growth and development were affected and it is not known if this overexpression had any influence on translation. The generation of viable transgenic lines overexpressing specific CK2 subunits opens the door for a number of experiments aimed at evaluating the *in vivo* effects of CK2 activity on translation.

It will also be important for future studies to evaluate transgenic knockouts/knockdown plants. Single T-DNA insertion mutants for CK2α1, CK2α2, and the four CK2β subunits show no obvious phenotype (Salinas et al., 2006); however, it is not known if these knockouts show subtle differences in translation or reduced multifactor complex formation. It is important that future studies develop double and triple mutants to further evaluate the role of CK2 in plants. One of the more disappointing moments in this research came with the discovery of a CK2α antisense

transgenic *A. thaliana* line, which had been previously generated at the University of Texas in 1999 to evaluate the role of CK2 in the circadian clock (Lee et al., 1999b). These transformants, which exhibited significant reductions in both CK2 activity and leaf size, would have been an ideal tool for this study. Unfortunately, seeds from these transformants could not be located.

Once transgenic plants have been generated, a number of experiments could be performed to evaluate the *in vivo* phosphorylation of initiation factors by CK2. An analysis of the phosphorylation state of eIFs in wild-type and transgenic plants could potentially provide further insight into *in vivo* phosphorylation. Initiation factors can be pulled-down from plant extracts and visualized with Pro-Q Diamond phosphoprotein stain (Invitrogen) to evaluate their phosphorylation status. The co-immunoprecipitation of factors from these extracts could also provide a comparison of multifactor complex formation in transgenic and wild-type plants. Due to the wide variety of substrates phosphorylated by CK2, it is likely that these transgenic plants will provide insight into a number of other plant cell signaling pathways.

### 5.2.2. CK2 Phosphorylation of eIF2 and eIF3

For the scope of this study, mutagenesis of CK2 phosphorylation sites in the multi-subunit factors eIF3 and eIF2 was not practical; however, the further studies aimed at these factors should provide further insight into the role CK2 plays in regulating translation initiation. The phosphorylation of eIF3c by CK2 is highly complex, and merits further investigation. A growing body of evidence suggests that the 13-subunit eIF3 complex may function as the central hub in the coordinating the events of translation

initiation (Hinnebusch, 2006). CK2 phosphorylation sites in the eIF3c N-terminus appear to be conserved in yeast, plants, and mammals; however, this research represents the first data to suggest these phosphorylation sites play a role in mediating interactions of multifactor complex components. Cloning procedures are currently underway to generate polycistronic wheat eIF3 using BioBricks. In conjunction with this project, a mutant eIF3c CK2 phosphorylation site knockout could be generated to further evaluate the role of eIF3c phosphorylation. The eIF3 complex prevents premature binding of the ribosomal subunits, enhances eIF2-GTP-Met-tRNA$_i$ ternary complex formation, directs the interactions of multiple eIFs during their assembly into pre-initiation complexes, improves mRNA recruitment to the 43S pre-initiation complex, and participates in both scanning and AUG recognition (Hinnebusch, 2006). Due to the extensive number of phosphorylation sites seen in eIF3c, it is possible that these modifications could influence the activity of eIF3 far beyond multifactor complex formation.

Analysis of wheat eIF3 complex phosphorylation initially suggested that only the eIF3c subunit was phosphorylated by CK2; however, following treatment with phosphatase (CIP) an additional phosphorylation site appeared at a molecular weight just greater than that of eIF3c (data not shown). While the identity of this "new" phosphorylation event has not been confirmed, it is suspected that this represents wheat eIF3b phosphorylation. *In vivo* phosphorylation sites have been identified in both *A. thaliana* and human eIF3b at consensus CK2 phosphorylation sites (Damoc et al., 2006; Heazlewood et al., 2008). Intriguingly, the phosphorylation site in *A. thaliana* eIF3b was located in the C-terminus at T685, a domain that in the yeast eIF3b (PRT1) is responsible for binding eIF3i (TIF34) and eIF3g (TIF35). In light of the role CK2

phosphorylation is suspected to play in multifactor complex formation, it is tempting to speculate a potential role for CK2 in the assembly of eIF3 complexes. Further investigation is needed to determine if the eIF3c subunit is truly the only eIF3 subunit phosphorylated by CK2 subunits. The eIF3 complex being generated by Biobricks will be useful for studying this hypothesis.

The role of CK2 phosphorylation in regulating eIF2 activity has yet to be fully investigated. Although native wheat CK2α does not affect the ability of eIF2 to form a ternary complex with Met-tRNA$_i^{Met}$ (Browning et al., 1985), there is reason to speculate that this may not be the case with CK2 holoenzymes. The phosphorylation of wheat eIF2β subunits by CK2 requires holoenzyme formation, and in isolation CK2α isoforms only phosphorylate eIF2α subunits (Chapter 2). Thus, the complete phosphorylation of eIF2 by CK2 would not have been evaluated in earlier studies. To properly investigate the influence of CK2 on eIF2 ternary complex formation, it will be necessary to perform future ternary complex formation assays in the presence of a variety of CK2 isoforms. In addition, little is known about the interaction of subunits within the plant eIF2 trimer. Based on modeling studies, it is generally assumed that the eIF2α and eIF2β subunits do not interact, but each binds to the intermediary eIF2γ subunit. Plant eIF2 appears to be somewhat unique compared to other forms of eukaryotic eIF2 (Browning, 2004), and it is not clear if its subunits are assembled in a similar manner. The assembly of these complexes represents a viable goal for future studies. Recombinant wheat eIF2β (p38) has been previously cloned and expressed (Metz and Browning, 1997), and was used extensively in this work for phosphorylation and binding assays. As part of this work, recombinant eIF2 α and γ-subunits were cloned and expressed; however a proper

investigation into the interaction of these recombinant subunits and the influence of CK2 phosphorylation has yet to take place. In addition, this study has produced an eIF2α mutant S318A that is not phosphorylated by CK2. Cloning is currently underway to assemble polycistronic recombinant wheat eIF2 from BioBricks. With these tools, future studies should be well equipped to provide further insight into the role of CK2 phosphorylation in regulating eIF2 activity.

# Appendix

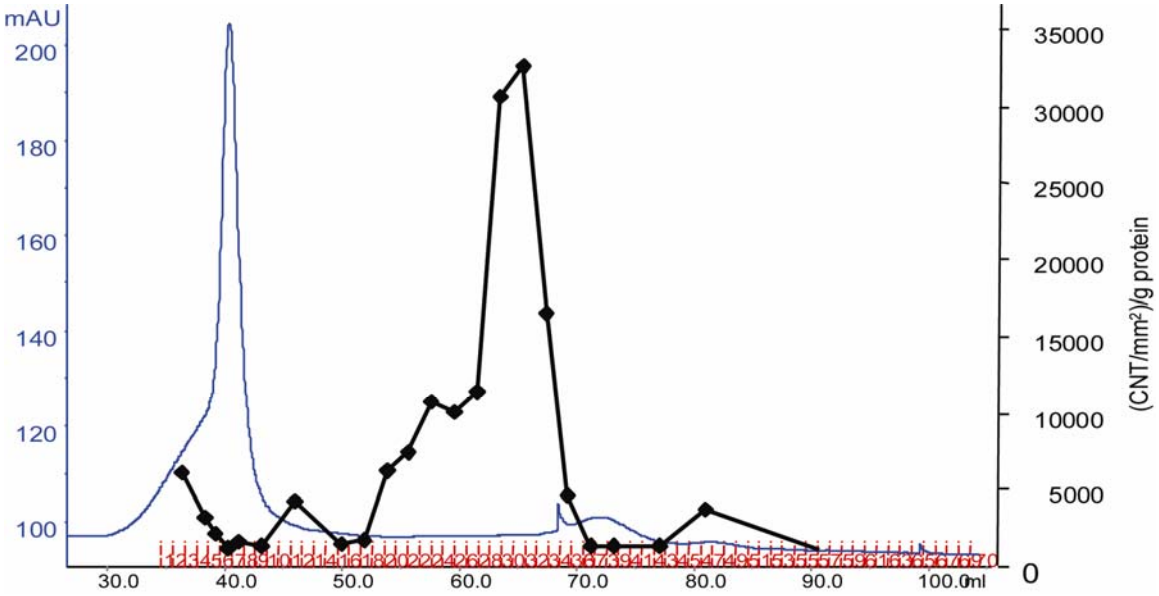



**Figure 2.10. S-200 HR gel filtration and evaluation of wheat germ kinase specific activity**

Fractions containg wheat germ kinase activity were analyzed by FPLC gel filtration using an Sephacryl S-200 HR column (GE Biosciences). The above graph includes both the spectrophotometer reading during elution (mAU; blue) and the specific activity of fractions (CNT/mm$^2$/g protein). To determine specific activity, protein concentrations were evaluated by Bradford assay, followed by kinase assay in reactions containing 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM [$\gamma$-$^{32}$P]ATP (~250 cpm/pmol), 1 µg of eIF3 and 5 µl of the elution fraction. Incubation was at 30 $^{o}$C for 20 min. The reaction was terminated by the addition of 4x SDS loading dye (2.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 0.1% bromophenol blue). Samples were then heated at 100°C for 5 min and loaded on a 12.5% SDS-PAGE. Following electrophoresis, gels were dried, and exposed to a phosphorimager screen (Molecular Dynamics) for 12 hours. The screen was then analyzed using Molecular Imager FX and Quantity One Software (BioRad) was used to quantify phosphorylation.

A.

gi|14317906 **Mass:** 39515 **Score:** 157 **Expect:** 6.2e-010 **Peptides matched:** 17

casein kinase II alpha [Triticum aestivum]

| Observed | Mr(expt) | Mr(calc) | Delta | Start | End | Miss | Ions | Peptide |
|---|---|---|---|---|---|---|---|---|
| 728.4508 | 727.4435 | 727.4592 | -0.0157 | 92 | 97 | 0 | --- | LLDIVR |
| 1259.7045 | 1258.6972 | 1258.7033 | -0.0061 | 6 | 16 | 0 | --- | VYADVNVVRPK |
| 1274.6526 | 1273.6453 | 1273.6163 | 0.0290 | 302 | 311 | 1 | --- | YDHQDRLTAR |
| 1323.7070 | 1322.6997 | 1322.7306 | -0.0309 | 92 | 102 | 1 | --- | LLDIVRDQHSK |
| 1425.7903 | 1424.7830 | 1424.7809 | 0.0021 | 79 | 91 | 0 | --- | ILQNLCGGPNIVK |
| 1425.7903 | 1424.7830 | 1424.7809 | 0.0021 | 79 | 91 | 0 | 50 | ILQNLCGGPNIVK |
| 1461.7317 | 1460.7244 | 1460.7234 | 0.0010 | 312 | 323 | 0 | --- | EAMAHPYFLQVR |
| 1461.7317 | 1460.7244 | 1460.7234 | 0.0010 | 312 | 323 | 0 | 61 | EAMAHPYFLQVR |
| 1477.7332 | 1476.7259 | 1476.7183 | 0.0076 | 312 | 323 | 0 | --- | EAMAHPYFLQVR + Oxidation (HW) |
| 1477.7332 | 1476.7259 | 1476.7183 | 0.0076 | 312 | 323 | 0 | --- | EAMAHPYFLQVR + Oxidation (M) |
| 1702.8732 | 1701.8659 | 1701.8620 | 0.0039 | 151 | 164 | 0 | --- | DVKPHNVMIDHELR |
| 1702.8732 | 1701.8659 | 1701.8620 | 0.0039 | 151 | 164 | 0 | --- | DVKPHNVMIDHELR |
| 1755.8135 | 1754.8062 | 1754.8110 | -0.0048 | 45 | 59 | 0 | --- | YSEVFEGINVNNNEK |
| 1830.9673 | 1829.9600 | 1829.9570 | 0.0030 | 151 | 165 | 1 | --- | DVKPHNVMIDHELRK |
| 1830.9673 | 1829.9600 | 1829.9570 | 0.0030 | 151 | 165 | 1 | --- | DVKPHNVMIDHELRK |
| 1845.8688 | 1844.8615 | 1844.8481 | 0.0134 | 225 | 239 | 0 | --- | EPFFYGHDNHDQLVK |
| 1940.9398 | 1939.9325 | 1939.9274 | 0.0051 | 43 | 59 | 1 | 16 | GKYSEVFEGINVNNNEK |

**No match to:** 818.4203, 818.4203, 969.6097, 969.6097, 1002.5372, 1053.6165, 1053.6165, 1108.5160, 1108.5160, 1159.6375, 1159.6375, 1175.6403, 1175.6403, 1219.6721, 1219.6721, 1247.6738, 1266.6788, 1267.7023, 1267.7023, 1273.6725, 1281.6740, 1283.6952, 1285.7047, 1289.7119, 1295.6943, 1295.6943, 1297.6923, 1299.6897, 1301.6952, 1305.6453, 1309.6947, 1311.6877, 1315.6946, 1315.6946, 1316.7070, 1321.6287, 1325.7089, 1331.7057, 1343.6840, 1368.6608, 1371.7000, 1377.7313, 1384.7190, 1384.7190, 1398.7070, 1400.7095, 1406.7124, 1412.7223, 1413.7189, 1421.7870, 1422.7334, 1428.7242, 1438.6985, 1439.7445, 1440.7817, 1487.7837, 1503.7327, 1503.7327, 1688.8422, 1735.7783, 1735.7783, 1759.9462, 1759.9462, 1760.9459, 1816.9515, 1883.9365, 1883.9365, 1920.9086, 1920.9086

B.

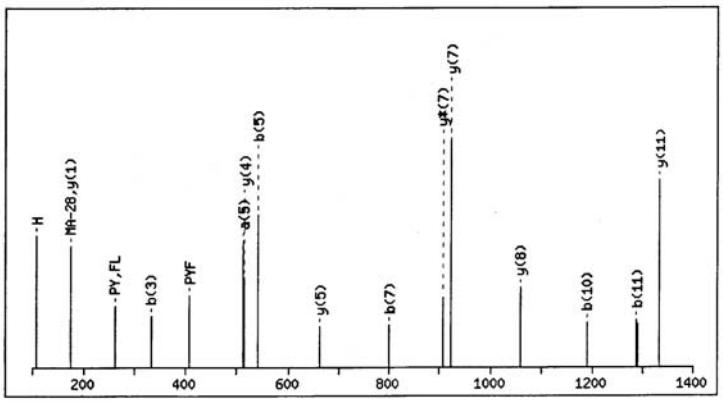



**Figure 2.11. Identification of wheat germ kinase 37-kDa band as CK2α by mass spectrometry**

The wheat germ kinase previously characterized by Yan & Tao (1982) was unambiguously identified by mass spectrometry as casein kinase II alpha (CK2α; Figure 2.1, band 1). CK2α was purified from wheat germ by size exclusion and ion-exchange chromatography. The 37-kDa band in the purified sample was isolated by SDS-PAGE, and digested with trypsin as described in Chapter 2. A. Mascot search results identify a high probability match with gi|14317906|dbj|BAB59136.1. This match represents 31% sequence coverage with 17 matched peptides. MS/MS spectra were obtained for peptides ILQNLCGGPNIVK, EAMAHPYFLQVR, and GKYSEVFEFINVNNNEK. B. MS/MS fragmentation of tryptic fragment EAMAHPYFLQVR. Using the 18 most intense peaks from fragmentation of the wheat germ kinase peptide with mass 1460.72, 19/155 fragment ions were identified.

**Table 2.2. Primers used in cloning**

| Primer | Sequence (5'-3') |
|---|---|
| At-CK2A1 5' NdeI | ATA*CATATG*TCGAAAGCTCGTGTGTACACCG |
| At-CK2A1 3' BamHI | AGT*GGATCC*TCATTGACTTCTCATTCTGCTGG |
| At-CK2A1 5' BspHI | AGCTAGCT*TCATGA*TGTCGAAAGCTCGTGTG |
| At-CK2A1 3' XhoI | GCT*CTCGAG*TTGACTTCTCATTCTGC |
| At-CK2A2 5' NcoI | ACTG*CCATGGG*TTCGAAAGCTCGTG |
| At-CK2A2 3' XhoI | AGCT*CTCGAG*TTGAGTCCTCATTCTGC |
| At-CK2A3 5' NdeI | CCA*CATATG*TCGAAAGCTAGGGTTTATAC |
| At-CK2A3 3' NotI | CGAGT*GCGGCCGC*CTGAGTTCGTAGTCTGCTGC |
| At-CK2Acp 5' NdeI | GACT*CATATG*GCCTTAAGGCCTTGTAC |
| At-CK2Acp 3' XhoI | TGTG*CTCGAG*CTGGCTGCGCGGCGTACGG |
| | |
| At-CK2B1 5' NcoI | ACTG*CCATGGG*TTATAGAGACAGAGG |
| At-CK2B1 3' XhoI | GGAC*CTCGAG*CGGTTTGTGTAATTTGAACC |
| At-CK2B2 5' NcoI | AGCT*CCATGG*GTTATAGGGAGAGAGGTA |
| At-CK2B2 3' XhoI | AGCT*CTCGAG*CGGCTTGTGTAGCTT |
| At-CK2B3 5' NcoI | AGCT*CCATGG*GTTACAAGGAACGTAGTGGA |
| At-CK2B3 3' XhoI | GAT*CTCGAG*TGGTTTGTGTACCTTGAAGCC |
| At-CK2B4 5' NcoI | GCAC*CCATGG*ATGTACAAGGATCGGAGTGG |
| At-CK2B4 3' XhoI | TGTG*CTCGAG*TTGTTTGTTGTGTACCTTAAAGCC |
| | |
| At-HD2beta 5' NcoI | AGCTAGCT*CCATGG*AGTTCTGGGGAGTTGCGG |
| At-HD2beta 3' XhoI | AGCT*CTCGAG*AGCTCTACCCTTTCCCTT |
| | |
| Ta-eIF2alpha 5' NdeI | CAGC*CATATG*GCGAACCTCGAGTGC |
| Ta-eIF2alpha 3' BamEco | *GAATTCGGATCC*TTAGTCCGCATGGAC |
| Ta-eIF3c 5' NcoI | AGCT*CCATGG*CGTCTCTGTTTTTGGGGAC |
| Ta-eIF3c 3' SacI | AGCT*GAGCTC*CTAATTTCTACCAGGCC |
| Ta-eIF3c 3' XhoI | AGCT*CTCGAG*ATTTCTACCAGGCCTG |

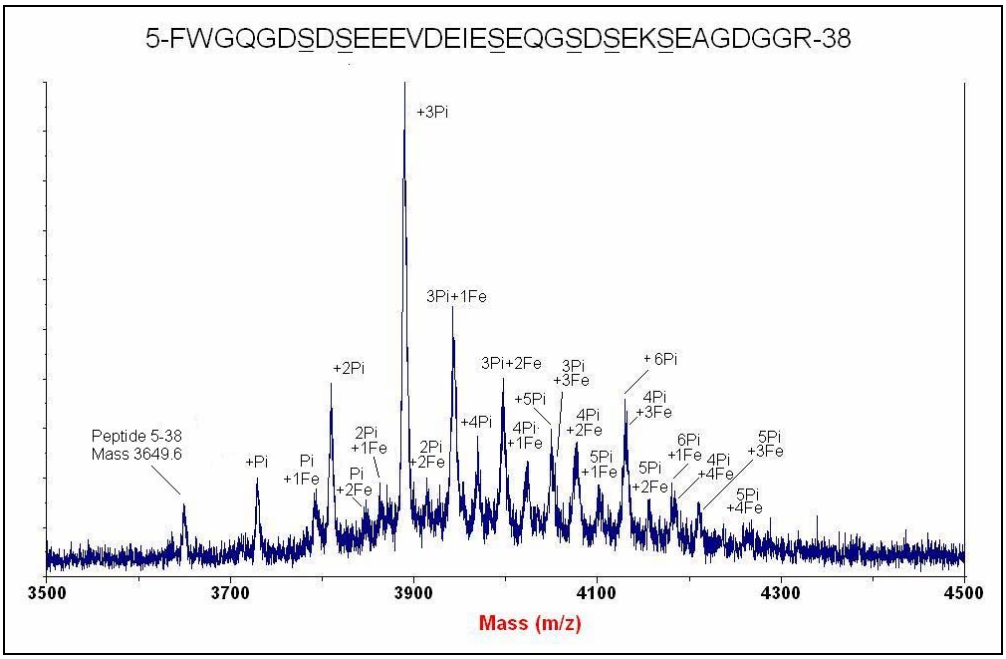
**Table 3.2. Primers used in mutagenesis of eIF2α and eIF5.**

| Primer | Sequence (5'-3') |
|---|---|
| Ta-eIF2alpha 5' NdeI | CAGC*CATATG*GCGAACCTCGAGTGC |
| Ta-eIF2alpha 3' BamEco | GAATTC*GGATCC*TTAGTCCGCATGGAC |
| Ta-eIF2alpha S318A F | TTGCTGGTGATGATGACGCTGAAGATGAGGAAGATAC |
| Ta-eIF2alpha S318A R | GTATCTTCCTCATCTTCAGCGTCATCATCACCAGCAA |
| | |
| Ta-eIF5 5' Nde I | GCT*CCATATG*GCGCTGCAAAACATTGG |
| Ta-eIF5 3' BamHI | GAGC*GGATCC*TCACTCCTCGTCGGACTCGGC |
| Ta-eIF5 S209A F | AGAAGGGTGCTGGGGGCGCTGATGAGGAACATGTCT |
| Ta-eIF5 S209A R | AGACATGTTCCTCATCAGCGCCCCCAGCACCCTTCT |
| Ta-eIF5 T240A F | ATGATGTACAGTGGGCGGCTGACACGTCAGCAGAGGC |
| Ta-eIF5 T240A R | GCCTCTGCTGACGTGTCAGCCGCCCACTGTACATCAT |
| Ta-eIF5 S451A F | CCAGAGCGCCGAGGCCGACGAGGAGTGAGG |
| Ta-eIF5 S451-A R | CCTCACTCCTCGTCGGCCTCGGCGCTCTGG |

**Table 3.3 Mass shifts in hexaphosphopeptide identified in wheat eIF3c-NTD**

| Phosphates | Irons Added (+53 Da) | | | | |
|---|---|---|---|---|---|
| Added (+80 Da) | 0 | 1 | 2 | 3 | 4 |
| 0 | **3649.6*** | | | | |
| 1 | 3729.6 | 3782.6 | 3835.6 | | |
| 2 | 3809.6 | 3862.6 | 3915.6 | 3968.6 | |
| 3 | 3889.6 | 3942.6 | 3995.6 | 4048.6 | 4101.6 |
| 4 | 3969.6 | 4022.6 | 4075.6 | 4128.6 | 4181.6 |
| 5 | 4049.6 | 4102.6 | 4155.6 | 4208.6 | 4261.6 |
| 6 | 4129.6 | 4182.6 | | | |

* Mass of unmodified peptide *5*FWGQGDSDSEEEVDEIESEQGSDSEKSEAGDGGR*38*.



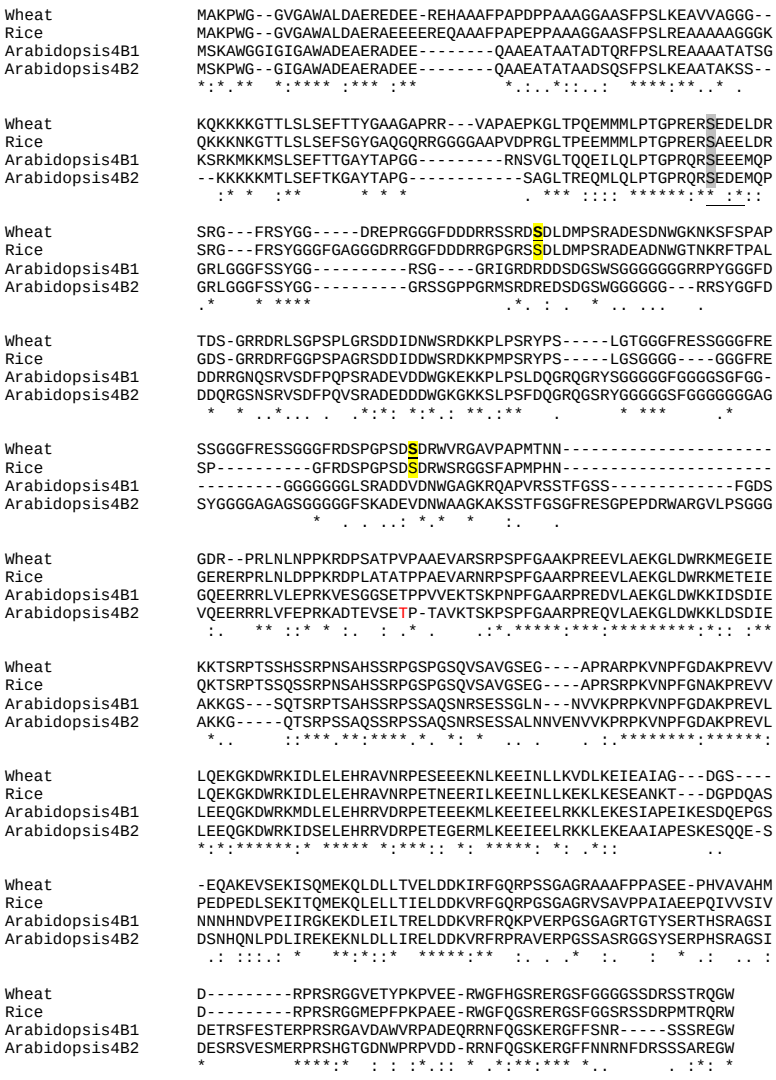



**Figure 3.11. Alignment of plant eIF4B protein sequences**

Multiple sequence alignment of eIF4B was performed using Clustal 2.0.2. Shown are the sequences for the wheat eIF4B (*AAC28254.1*), rice eIF4B (*Os04g0480100*), *A. thaliana* eIF4B1 (*AAF05869.1*), *A. thaliana* eIF4B2 (*AAF27287.1*). Recombinant wheat eIF4B is phosphorylated *in vitro* by CK2 phosphorylation at S140 and S241. These phosphorylation sites are conserved in rice, yet are absent from either *A. thaliana* eIF4B. The CK2 phosphorylation sites identified in this study are indicated in bold and underline. The most favorable consensus CK2 phosphorylation site identified by Scansite (S106 indicated in grey; www.scansite.mit.edu) was not observed in the MS analysis. This phosphorylation site does appear to be conserved across plants; however, no evidence of its *in vitro* phosphorylation was produced by this study.

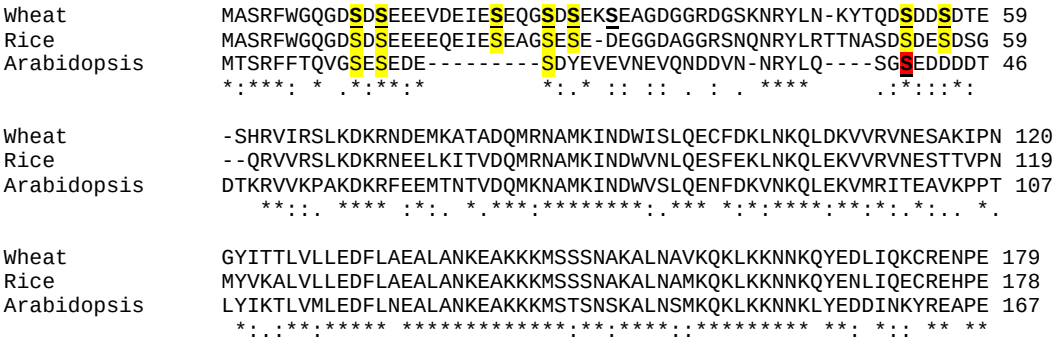



**Figure 3.12. Alignment of plant eIF3c N-terminal amino acids**

Multiple sequence alignment of eIF3c N-terminal residues. Shown are the sequences for the N-terminus of wheat (*TC235391*), rice (*EAZ38556*), and *A. thaliana* (*AAC83464*) eIF3c. CK2 phosphorylation sites were identified in the N-terminus of recombinant wheat eIF3c-NTD (indicated in bold/underlined). Conserved consensus CK2 sites in the N-terminus eIF3c sequences are indicated. The *in vivo* phosphorylation of the *A. thaliana* eIF3c N-terminus at consensus CK2 site S40 was recently observed by large-scale phosphoproteonomic analysis (indicated in red; (Heazlewood et al., 2008).

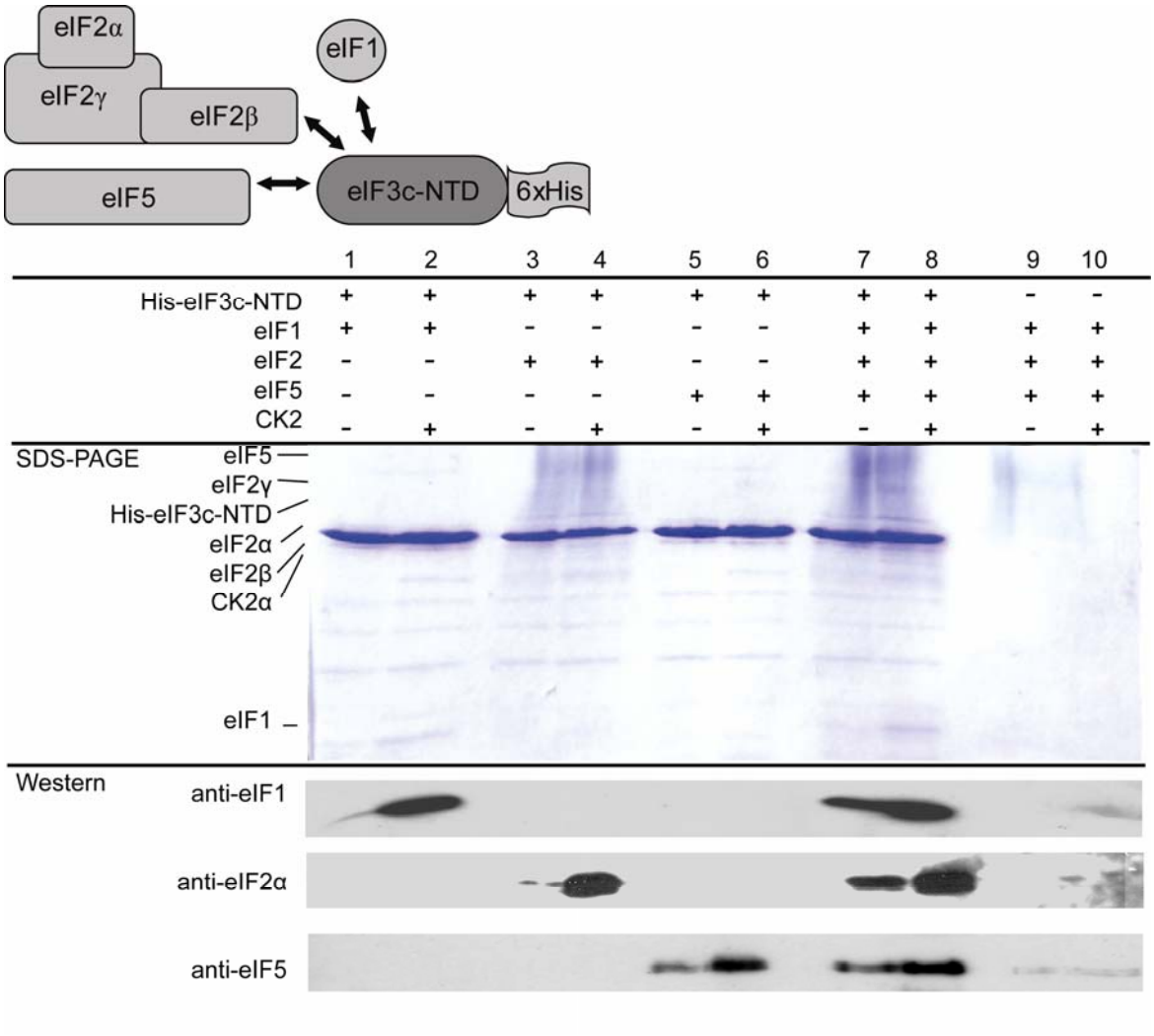



**Figure 4.1. CK2 enhances interaction of MFC components with eIF3c NTD *in vitro***

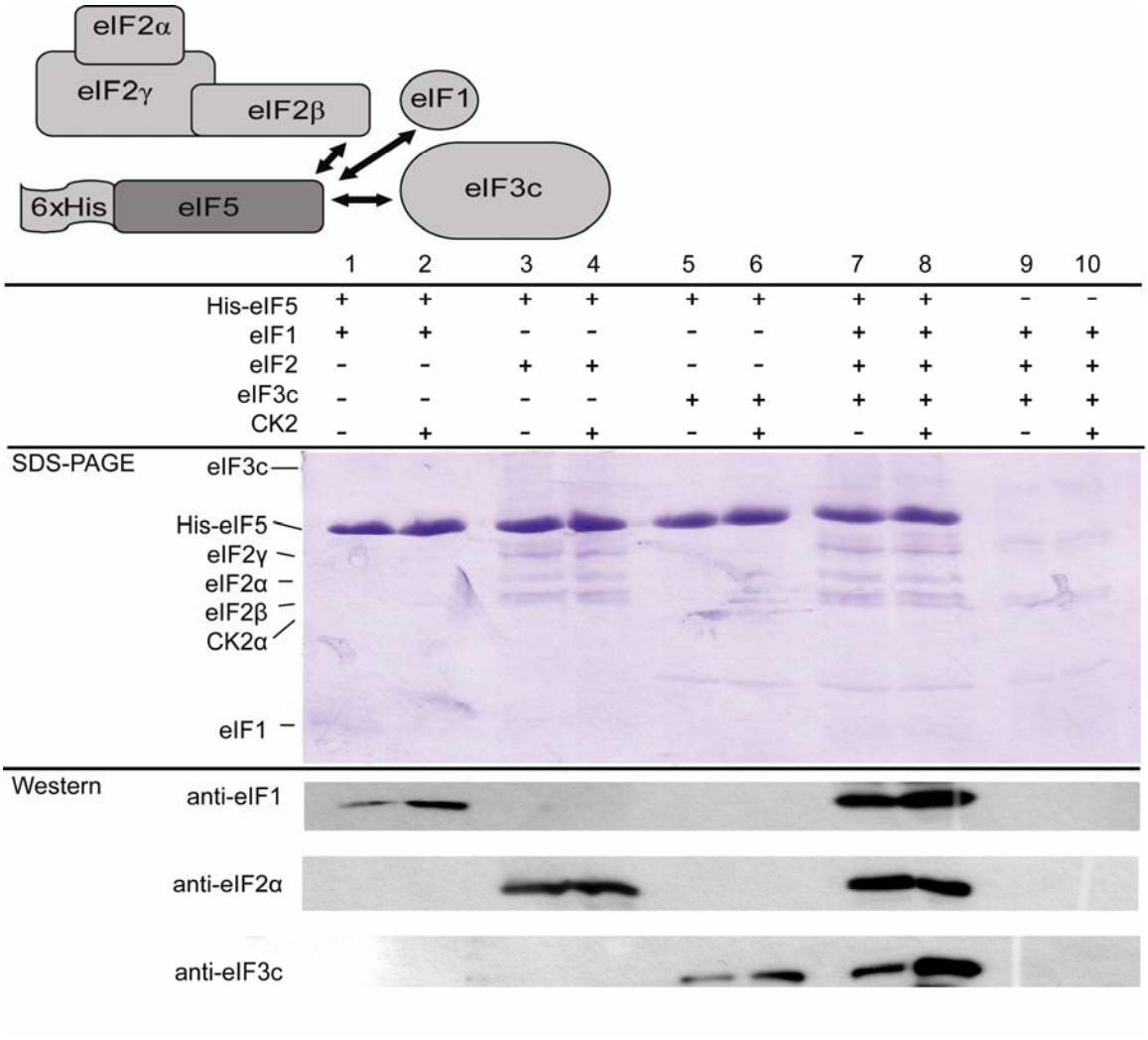



**Figure 4.2. CK2 enhances interaction of MFC components with His-eIF5 *in vitro***

# References


Abramczyk O, Zien P, Zielinski R, Pilecki M, Hellman U, Szyszka R (2003) The protein kinase 60S is a free catalytic CK2 alpha ' subunit and forms an inactive complex with superoxide dismutase SOD1. Biochem Biophys Res Commun **307**: 31-40

Allende JE, Allende CC (1995) Protein Kinases .4. Protein-Kinase Ck2 - An Enzyme with Multiple Substrates and A Puzzling Regulation. FASEB Journal **9**: 313-323

Alone PV, Dever TE (2006) Direct binding of translation initiation factor eIF2 gamma-G domain to its GTPase- activating and GDP-GTP exchange factors eIF5 and eIF2B epsilon. J Biol Chem **281**: 12636-12644

Asano K, Clayton J, Shalev A, Hinnebusch AG (2000) A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator $tRNA^{Met}$ is an important translation initiation intermediate in vivo. Genes & Dev **14**: 2534-2546

Asano K, Krishnamoorthy T, Phan L, Pavitt GD, Hinnebusch AG (1999) Conserved bipartite motifs in yeast eIF5 and eIF2Be, GTPase- activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. EMBO J **18**: 1673-1688

Asano K, Phan L, Anderson J, Hinnebusch AG (1998) Complex formation by all five homologues of mammalian translation initiation factor 3 subunits from yeast *Saccharomyces cerevisiae*. J Biol Chem **273**: 18573-18585

Asano K, Phan L, Valásek L, Schoenfeld LW, Shalev A, Clayton J, Nielsen K, Donahue TF, Hinnebusch AG (2001a) A multifactor complex of eIFI, cIF2, eIF3, eIF5, and $tRNA_i^{Met}$ promotes initiation complex assembly and couples GTP hydrolysis to AUG recognition. Cold Spring Harbor Symp Quant Biol **66**: 403-415

Asano K, Shalev A, Phan L, Nielsen K, Clayton J, Valásek L, Donahue TF, Hinnebusch AG (2001b) Multiple roles for the C-terminal domain of eIF5 in translation initiation complex assembly and GTPase activation. EMBO J **20**: 2326-2337

Bandyopadhyay A, Lakshmanan V, Matsumoto T, Chang EC, Maitra U (2002) Moe1 and spInt6, the fission yeast homologues of mammalian translation initiation factor 3 Subunits p66 (eIF3d) and p48 (eIF3e), respectively, are required for stable association of eIF3 Subunits. J Biol Chem **277**: 2360-2367

Benaim G, Villalobo A (2002) Phosphorylation of calmodulin - Functional implications. Eur J Biochem **269**: 3619-3631

Benkowski LA, Ravel JM, Browning KS (1995a) Development of an *in vitro* translation system from wheat germ that is dependent upon the addition of eukaryotic initiation factor 2. Anal Biochem **232**: 140-143

Benkowski LA, Ravel JM, Browning KS (1995b) mRNA binding properties of wheat germ protein synthesis initiation factor 2. Biochem Biophys Res Commun **214**: 1033-1039

Beretta L, Gingras AC, Svitkin YV, Hall MN, Sonenberg N (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J **15**: 658-664

Bidwai AP, Reed JC, Glover CV (1995) Cloning and disruption of CKB1, the gene encoding the 38-kDa beta subunit of Saccharomyces cerevisiae casein kinase II (CKII). Deletion of CKII regulatory subunits elicits a salt-sensitive phenotype. J Biol Chem **270**: 10395-10404

Blau J (2003) A new role for an old kinase: CK2 and the circadian clock. Nat Neurosci **6**: 208-210

Bolanos-Garcia VM, Fernandez-Recio J, Allende JE, Blundell TL (2006) Identifying interaction motifs in CK2 beta - a ubiquitous kinase regulatory subunit. Trends Biochem Sci **31**: 654-661

Boldyreff B, Issinger OG (1997) A-Raf kinase is a new interacting partner of protein kinase CK2 beta subunit. FEBS Lett **403**: 197-199

Boldyreff B, Meggio F, Pinna LA, Issinger OG (1994) Protein-Kinase Ck2 Structure - Function Relationship - Effects of the Beta-Subunit on Reconstitution and Activity. Cellular & Molecular Biology Research **40**: 391-399

Boldyreff B, Mietens U, Issinger OG (1996) Structure of protein kinase CK2: Dimerization of the human beta-subunit. FEBS Lett **379**: 153-156

Borman AM, Michel YM, Kean KM (2000) Biochemical characterisation of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end. Nucleic Acids Res **28**: 4068-4075

Browning KS (1996) The plant translational apparatus. Plant Molecular Biology **32**: 107-144

Browning KS (2004) Plant translation initiation factors: it is not easy to be green. Biochem Soc Trans **32**: 589-591

Browning KS, Fletcher L, Lax SR, Ravel JM (1989) Evidence that the 59-kDa protein synthesis initiation factor from wheat germ is functionally similar to the 80-kDa initiation factor 4B from mammalian cells. J Biol Chem **264**: 8491-8494

Browning KS, Gallie DR, Hershey JWB, Hinnebusch AG, Maitra U, Merrick WC, Norbury C (2001) Unified nomenclature for the subunits of eukaryotic initiation factor 3. Trends Biochem Sci **26**: 284-284

Browning KS, Webster C, Roberts JKM, Ravel JM (1992) Identification of an Isozyme Form of Protein Synthesis Initiation Factor 4F in Plants. J Biol Chem **267**: 10096-10100

Browning KS, Yan TFJ, Lauer SJ, Aquino LA, Tao M, Ravel JM (1985) Phosphorylation of wheat germ initiation factors and ribosomal proteins. Plant Physiol **77**: 370-373

Burks EA, Bezerra PP, Le H, Gallie DR, Browning KS (2001) Plant initiation factor 3 subunit composition resembles mammalian initiaton factor 3 and has a novel subunit. J Biol Chem **276**: 2133-2131

Byrd MP, Zamora M, Lloyd RE (2002) Generation of multiple isoforms of eukaryotic translation initiation factor 4GI by use of alternate translation initiation codons. Mol Cell Biol **22**: 4499-4511

Chang LY, Yang WY, Browning K, Roth D (1999) Specific *in vitro* phosphorylation of plant eIF2a by eukaryotic eIF2a kinases. Plant Molecular Biology **41**: 363-370

Cheng S, Gallie DR (2006) Wheat eukaryotic initiation factor 4B organizes assembly of RNA and eIFiso4G, eIF4A, and PABP. J Biol Chem **281**: 24351-24364.

Cigan AM, Pabich EK, Feng L, Donahue TF (1989) Yeast translation initiation suppressor *sui2* encodes the a subunit of eukaryotic initiation factor 2 and shares sequence identity with the human a subunit. Proc Natl Acad Sci USA **86**: 2784-2788

Conte MR, Kelly G, Babon J, Sanfelice D, Youell J, Smerdon SJ, Proud CG (2006) Structure of the Eukaryotic Initiation Factor (eIF) 5 Reveals a Fold Common to Several Translation Factors. Biochemistry **45**: 4550-4558

Damoc E, Fraser C, Doudna J, Leary J (2006) Structural characterization and identification of post-translational modifications of human eukaryotic initiation factor 3 (eIF3) by FTICR mass spectrometry. FASEB Journal **20**: A528-A528

Daniel X, Sugano S, Tobin EM (2004) CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in Arabidopsis. Proc Natl Acad Sci U S A **101**: 3292-3297

Das S, Ghosh R, Maitra U (2001) Eukaryotic translation initiation factor 5 functions as a GTPase- activating protein. J Biol Chem **276**: 6720-6726

Das S, Maitra U (2000) Mutational analysis of mammalian translation initiation factor 5 (eIF5): Role of interaction between the b subunit of eIF2 and eIF5 in eIF5 function in vitro and in vivo. Mol Cell Biol **20**: 3942-3950

De Marchis ML, Giorgi A, Schinna ME, Bozzoni I, Fatica A (2005) Rrp15p, a novel component of pre-ribosomal particles required for 60S ribosome subunit maturation. RNA Publ RNA Soc **11**: 495-502

Deprost D, Yao L, Sormani R, Moreau M, Leterreux G, Nicolai M, Bedu M, Robaglia C, Meyer C (2007) The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. Embo Reports **8**: 864-870

Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F, Pinna LA, Ruzzene M (2005) Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death and Differentiation **12**: 668-677

Donahue TF, Cigan AM, Pabich EK, Valavicius BC (1988) Mutations at a Zn(II) Finger Motif in the Yeast eIF-2B Gene Alter Ribosomal Start-Site Selection during the Scanning Process. Cell **54**: 621-632

Duncan RF, Hershey JWB (1987) Initiation Factor Protein Modifications and Inhibition of Protein Synthesis. Mol Cell Biol **7**: 1293-1295

Dyer JR, Michel S, Lee W, Castellucci VF, Wayne NL, Sossin WS (2003) An activity-dependent switch to cap-independent translation triggered by eIF4E dephosphorylation. Nat Neurosci **6**: 219-220

Erickson FL, Hannig EM (1996) Ligand interactions with eukaryotic translation initiation factor 2: Role of the gamma-subunit. EMBO J **15**: 6311-6320

Espunya MC, Lopez-Giraldez T, Hernan I, Carballo M, Martinez MC (2005) Differential expression of genes encoding protein kinase CK2 subunits in the plant cell cycle. J Exp Bot **56**: 3183-3192

Feng L, Yoon H, Donahue TF (1994) Casein kinase II mediates multiple phosphorylation of *Saccharomyces cerevisiae* eIF-2a (encoded by *SUI2*), which is required for optimal eIF-2 function in *S. cerevisiae*. Mol Cell Biol **14**: 5139-5153

Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C, Blenis J (2004) mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol **24**: 200-216

Fletcher CM, Pestova TV, Hellen CUT, Wagner G (1999) Structure and interactions of the translation initiation factor eIF1. EMBO J **18**: 2631-2637

Flynn A, Vries RGJ, Proud CG (1997) Signalling pathways which regulate eIF4E. Biochem Soc Trans **25**: 192S-192S

Fraser CS, Berry KE, Hershey JW, Doudna JA (2007) eIF3j Is Located in the Decoding Center of the Human 40S Ribosomal Subunit. Mol Cell **26**: 811-819

Gallie DR (2007) Translational Control in Plants and Chloroplasts. In Mathews, M.B., Sonenberg, N., and Hershey, J.W.B., editors *Translational Control in Biology and Medicine*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 747-774

Gallie DR, Browning KS (2001) eIF4G functionally differs from eIFiso4G in promoting internal initiation, cap-independent translation, and translation of structured mRNAs. J Biol Chem **276**: 36951-36960

Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes & Dev **12**: 502-513

Gingras AC, Raught B, Sonenberg N (2004) mTOR signaling to translation. Curr Top Microbiol Immunol **279**: 169-197

Graham KC, Litchfield DW (2000) The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. J Biol Chem **275**: 5003-5010

Gupta NK, Roy AL, Nag MK, Kinzy TG, MacMillan S, Hileman RE, Dever TE, Wu S, Merrick WC, Hershey JWB (1990) New insights into an old problem: ternary complex (Met-tRNAf.eIF2.GTP) formation in animal cells. In McCarty, J.E.G. and Tuite, M.F., editors *Post-Transcriptional Control of Gene Expression*, Springer-Verlag, Berlin. pp. 521-526

Hagemann C, Kalmes A, Wixler V, Wixler L, Schuster T, Rapp UR (1997) The regulatory subunit of protein kinase CK2 is a specific A-Raf activator. FEBS Lett **403**: 200-202

Hannig EM, Cigan AM, Freeman BA, Kinzy TG (1993) *GCD11*, a negative regulator of *GCN4* expression, encodes the gamma subunit of eIF-2 in *Saccharomyces cerevisiae*. Mol Cell Biol **13**: 506-520

Harris TE, Chi A, Shabanowitz J, Hunt DF, Rhoads RE, Lawrence JC (2006) mTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. EMBO J **25**: 1659-1668

Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev **18**: 1926-1945

Heazlewood JL, Durek P, Hummel J, Selbig J, Weckwerth W, Walther D, Schulze WX (2008) PhosPhAt: a database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor. Nucleic Acids Res **36**: D1015-D1021

Hershey JWB (1989) Protein phosphorylation controls translation rates. J Biol Chem **264**: 20823-20826

Hershey JWB, Merrick WC (2000) Pathway and Mechanism of Initiation of Protein Synthesis. In Sonenberg, N., Hershey, J.W.B., Mathews, M.B. *Translational Control of Gene* Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp.33-88

Hinnebusch AG (2006) eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem Sci **31**: 553-562

Hinnebusch AG, Dever TE, Asano K (2007) Mechanism of Translation Initiation in the Yeast *Saccharomyces cerevisiae.* In Mathews, M.B., Sonenberg, N., and Hershey, J.W.B., editors *Translational Control in Biology and Medicine*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 225-268

Holz MK, Ballif BA, Gygi SP, Blenis J (2005) mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events. Cell **123**: 569-580

Homma MK, Homma Y (2005) Regulatory role of CK2 during the progression of cell cycle. Mol Cell Biochem **274**: 47-52

Homma MK, Wada I, Suzuki T, Yamaki J, Krebs EG, Homma Y (2005) CK2 phosphorylation of eukaryotic translation initiation factor 5 potentiates cell cycle progression. Proc Natl Acad Sci U S A **102**: 15688-15693

Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champion A, Kreis M, Zhang SQ, Hirt H, Wilson C, Heberle-Bors E, Ellis BE, Morris PC, Innes RW, Ecker JR, Scheel D, Klessig DF, Machida Y, Mundy J, Ohashi Y, Walker JC (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science **7**: 301-308

Ingram GC, Waites R (2006) Keeping it together: co-ordinating plant growth. Current Opinion in Plant Biology **9**: 12-20

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ (1989) Calyculin-A and Okadaic Acid - Inhibitors of Protein Phosphatase-Activity. Biochem Biophys Res Commun **159**: 871-877

Janaki N, Krishna VM, Ramaiah KVA (1995) Phosphorylation of wheat germ initiation factor 2 (eIF-2) by *N*-ethylmaleimide-treated wheat germ lysates and by purified casein kinase II does not affect the guanine nucleotide exchange on eIF-2. Arch Biochem Biophys **324**: 1-8

Johnson LN, Noble MEM, Owen DJ (1996) Active and inactive protein kinases: Structural basis for regulation. Cell **85**: 149-158

Jonak C, Ligterink W, Hirt H (1999) MAP kinases in plant signal transduction. Cell Mol Life Sci **55**: 204-213

Karniol B, Yahalom A, Kwok S, Tsuge T, Matsui M, Deng XW, Chamovitz DA (1998) The *Arabidopsis* homologue of an eIF3 complex subunit associates with the COP9 complex. FEBS Lett **439**: 173-179

Khan MA, Goss DJ (2004) Phosphorylation states of translational initiation factors affect mRNA cap binding in wheat. Biochemistry **43**: 9092-9097

Kozak M (1978) How do eucaryotic ribosomes select initiation regions in messenger RNA? Cell **15**: 1109-1123

Kubinski K, Domanska K, Sajnaga E, Mazur E, Zielinski R, Szyszka R (2007) Yeast holoenzyme of protein kinase CK2 requires both beta and beta ' regulatory subunits for its activity. Mol Cell Biochem **295**: 229-236

Kyrpides NC, Woese CR (1998) Universally conserved translation initiation factors. Proc Natl Acad Sci USA **95**: 224-228

Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC (2001) Protein kinase CK2 in mammary gland tumorigenesis. Oncogene **20**: 3247-3257

Langland JO, Jin S, Jacobs BL, Roth DA (1995) Identification of a plant-encoded analog of PKR, the mammalian double-stranded RNA-dependent protein kinase. Plant Physiol **108**: 1259-1267

Langland JO, Langland LA, Browning KS, Roth DA (1996) Phosphorylation of plant eukaryotic initiation factor-2 by the plant-encoded double-stranded RNA-dependent protein kinase, pPKR, and inhibition of protein synthesis *in vitro*. J Biol Chem **271**: 4539-4544

Lax SR, Lauer SJ, Browning KS, Ravel JM (1986) Purification and Properties of Protein Synthesis Initiation and Elongation Factors from Wheat Germ. Methods Enzymol **118**: 109-128

Le H, Browning KS, Gallie DR (2000) The phosphorylation state of poly(A)-binding protein specifies its binding to poly(A) RNA and its interaction with eukaryotic initiation factor (eIF) 4F, eIFiso4F, and eIF4B. J Biol Chem **275**: 17452-17462

Lee Y, Lloyd AM, Roux SJ (1999) Antisense expression of the CK2 alpha-subunit gene in Arabidopsis. Effects on light-regulated gene expression and plant growth. Plant Physiol **119**: 989-1000

Ling J, Morley SJ, Traugh JA (2005) Inhibition of cap-dependent translation via phosphorylation of eIF4G by protein kinase Pak2. EMBO J **24**: 4094-4105

Litchfield DW (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. Biochem J **369**: 1-15

Llorens F, Duarri A, Sarro E, Roher N, Plana M, Itarte E (2005a) The N-terminal domain of the human eIF2-beta subunit and the CK2 phosphorylation sites are required for its function. Biochem J **394**: 227-236

Llorens F, Sarno S, Sarro E, Duarri A, Roher N, Meggio F, Plana M, Pinna LA, Itarte E (2005b) Cross talk between protein kinase CK2 and eukaryotic translation initiation factor eIF2beta subunit
96. Mol Cell Biochem **274**: 53-61

Lusser A, Brosch G, Loidl A, Haas H, Loidl P (1997) Identification of maize histone deacetylase HD2 as an acidic nucleolar phosphoprotein. Science **277**: 88-91

Ma SH, Musa T, Bag J (2006) Reduced stability of mitogen-activated protein kinase kinase-2 mRNA and phosphorylation of poly(A)-binding protein (PABP) in cells overexpressing PABP. J Biol Chem **281**: 3145-3156

Maag D, Fekete CA, Gryczynski Z, Lorsch JR (2005) A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. Mol Cell **17**: 265-275

Maiti T, Bandyopadhyay A, Maitra U (2003) Casein kinase II phosphorylates translation initiation factor 5 (elFS) in *Saccharomyces cerevisiae*. Yeast **20**: 97-108

Majumdar R, Bandyopadhyay A, Deng HT, Maitra U (2002) Phosphorylation of mammalian translation initiation factor 5 (eIF5) *in vitro* and *in vivo*. Nucleic Acids Res **30**: 1154-1162

Marin O, Meggio F, Pinna LA (1999) Structural features underlying the unusual mode of calmodulin phosphorylation by protein kinase CK2: A study with synthetic calmodulin fragments. Biochem Biophys Res Commun **256**: 442-446

Marintchev A, Wagner G (2004) Translation initiation: structures, mechanisms and evolution. Q Rev Biophys **37**: 197-284

Masutani M, Sonenberg N, Yokoyama S, Imataka H (2007) Reconstitution reveals the functional core of mammalian eIF3. EMBO J **26**: 3373-3383

Mayberry LK, Dennis MD, Leah AM, Ruud NK, Murphy PA, Campbell L, Browning KS (2007) Expression and Purification of Recombinant Wheat Translation Initiation Factors eIF1, eIF1A, eIF4A, eIF4B, eIF4F, eIF(iso)4F, and eIF5. Methods Enzymol **430**: 397-408

McInerney P, Mizutani T, Shiba T (2006) Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity in vitro and in vivo. Mol Microbiol **60**: 438-447

Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? FASEB Journal **17**: 349-368

Meggio F, Ruzzene M, Sarno S, Pagano MA, Pinna LA (2000) pCMB treatment reveals the essential role of cysteinyl residues in conferring functional competence to the regulatory subunit of protein kinase CK2. Biochem Biophys Res Commun **267**: 427-432

Methot N, Song MS, Sonenberg N (1996) A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol Cell Biol **16**: 5328-5334

Metz AM, Browning KS (1996) Mutational analysis of the functional domains of the large subunit of the isozyme form of wheat initiation factor eIF4F. J Biol Chem **271**: 31033-31036

Metz AM, Browning KS (1997) Assignment of the b-subunit of wheat eIF2 by protein and DNA sequence analysis and immunoanalysis. Arch Biochem Biophys **342**: 187-189

Metz AM, Wong KCH, Malmström SA, Browning KS (1999) Eukaryotic initiation factor 4B from wheat and *Arabidopsis thaliana* is a member of a multigene family. Biochem Biophys Res Commun **266**: 314-321

Minich WB, Balasta ML, Goss DJ, Rhoads RE (1994) Chromatographic resolution of *in vivo* phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: Increased cap affinity of the phosphorylated form. Proc Natl Acad Sci USA **91**: 7668-7672

Mishra NS, Tuteja R, Tuteja N (2006) Signaling through MAP kinase networks in plants. Arch Biochem Biophys **452**: 55-68

Miyamoto S, Patel P, Hershey JW (2005) Changes in ribosomal binding activity of eIF3 correlate with increased translation rates during activation of T lymphocytes **89**. J Biol Chem

Monzingo AF, Dhaliwal S, Dutt-Chaudhuri A, Lyon A, Sadow JH, Hoffman DW, Robertus JD, Browning KS (2007) The Structure of Eukaryotic Translation Initiation Factor-4E from Wheat Reveals a Novel Disulfide Bond. Plant Physiol **143**: 1504-1518

Morley SJ, Traugh JA (1990) Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters. J Biol Chem **265**: 10611-10616

Naegele S, Morley SJ (2004) Molecular cross-talk between MEK1/2 and mTOR signaling during recovery of 293 cells from hypertonic stress. J Biol Chem **279**: 46023-46034

Niefind K, Guerra B, Ermakowa I, Issinger OG (2001) Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. EMBO J **20**: 5320-5331

Niefind K, Guerra B, Pinna LA, Issinger OG, Schomburg D (1998) Crystal structure of the catalytic subunit of protein kinase CK2 from Zea mays at 2.1 angstrom resolution. EMBO J **17**: 2451-2462

Niefind K, Putter M, Guerra B, Issinger OG, Schomburg D (1999) CTP plus water mimic ATP in the active site of protein kinase CK2. Nature Struct Biol **6**: 1100-1103

Nika J, Yang WM, Pavitt GD, Hinnebusch AG, Hannig EM (2000) Purification and kinetic analysis of eIF2B from *Saccharomyces cerevisiae*. J Biol Chem **275**: 26011-26017

Nusspaumer G, Remacha M, Ballesta JPG (2000) Phosphorylation and N-terminal region of yeast ribosomal protein P1 mediate its degradation, which is prevented by protein P2. EMBO J **19**: 6075-6084

Ogrzewalla K, Piotrowski M, Reinbothe S, Link G (2002) The plastid transcription kinase from mustard (Sinapis alba L.) - A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. Eur J Biochem **269**: 3329-3337

Olsten MEK, Litchfield DW (2004) Order or chaos? An evaluation of the regulation of protein kinase CK2. Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire **82**: 681-693

Olsten MEK, Weber JE, Litchfield DW (2005) CK2 interacting proteins: Emerging paradigms for CK2 regulation? Mol Cell Biochem **274**: 115-124

Orru S, Aspesi A, Armiraglio M, Caterino M, Loreni F, Ruoppolo M, Santoro C, Dianzani I (2007) Analysis of the ribosomal protein S19 interactome. Molecular & Cellular Proteomics **6**: 382-393

Padmanabha R, Chenwu JLP, Hanna DE, Glover CVC (1990) Isolation, Sequencing, and Disruption of the Yeast Cka2 Gene - Casein Kinase-Ii Is Essential for Viability in Saccharomyces-Cerevisiae. Mol Cell Biol **10**: 4089-4099

Pagano MA, Sarno S, Poletto G, Cozza G, Pinna LA, Meggio F (2005) Autophosphorylation at the regulatory beta subunit reflects the supramolecular organization of protein kinase CK2. Mol Cell Biochem **274**: 23-29

Park GH, Lee YT, Bae YS (2001) Stimulation of human DNA topoisomerase II activity by its direct association with the beta subunit of protein kinase CKII. Molecules and Cells **11**: 82-88

Park HS, Browning KS, Hohn T, Ryabova LA (2004) Eucaryotic initiation factor 4B controls eIF3-mediated ribosomal entry of viral reinitiation factor. EMBO J **23**: 1381-1391

Patel J, McLeod LE, Vries RGJ, Flynn A, Wang XM, Proud CG (2002) Cellular stresses profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors. Eur J Biochem **269**: 3076-3085

Perales M, Portoles S, Mas P (2006) The proteasome-dependent degradation of CKB4 is regulated by the Arabidopsis biological clock. Plant J **46**: 849-860

Pestova TV, Borukhov SI, Hellen CUT (1998) Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature **394**: 854-859

Pestova TV, Kolupaeva VG (2002) The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev **16**: 2906-2922

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, Hellen CUT (2001) Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA **98**: 7029-7036

Pestova TV, Lomakin IB, Lee JH, Choi SK, Dever TE, Hellen CUT (2000) The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature **403**: 332-335

Pestova TV, Lorsch JR, Hellen CUT (2007) The Mechanism of Translation Initiation in Eukaryotes. In Mathews, M.B., Sonenberg, N., and Hershey, J.W.B., editors *Translational Control in Biology and Medicine*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 87-128

Peterson TR, Sabatini DM (2005) eIF3: a connecTOR of S6K1 to the translation preinitiation complex
90. Mol Cell **20**: 655-657

Phan L, Zhang XL, Asano K, Anderson J, Vornlocher HP, Greenberg JR, Qin J, Hinnebusch AG (1998) Identification of a translation initiation factor 3 (eIF3) core complex, conserved in yeast and mammals, that interacts with eIF5. Mol Cell Biol **18**: 4935-4946

Pinna LA (1994) A Historical View of Protein-Kinase Ck2. Cellular & Molecular Biology Research **40**: 383-390

Pinna LA (1997) Protein kinase CK2. International Journal of Biochemistry & Cell Biology **29**: 551-554

Pinna LA (2002) Protein kinase CK2: a challenge to canons. J Cell Sci **115**: 3873-3878

Pinna LA (2003) The raison D'Etre of constitutively active protein kinases: The lesson of CK2. Accounts Chem Res **36**: 378-384

Pinna LA (2006) 1954-2006: the long march of protein kinase CK2. FASEB Journal **20**: A499-A499

Pluemsampant S, Safronova OS, Nakahama K, Morita I (2008) Protein kinase CK2 is a key activator of histone deacetylase in hypoxia-associated tumors. International Journal of Cancer **122**: 333-341

Proud CG (1992) Phosphorylation in translational control. Curr Topics Cell Regulation **32**: 243-369

Proud CG (2007) Signalling to translation: how signal transduction pathways control the protein synthetic machinery. Biochem J **403**: 217-234

Pyronnet S, Imataka H, Gingras AC, Fukunaga R, Hunter T, Sonenberg N (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. EMBO J **18**: 270-279

Qin H, Raught B, Sonenberg N, Goldstein EG, Edelman AM (2003) Phosphorylation screening identifies translational initiation factor 4GII as an intracellular target of $Ca^{2+}$/Calmodulin- dependent protein kinase I. J Biol Chem **278**: 48570-48579

Raught B, Gingras AC, Gygi SP, Imataka H, Morino S, Gradi A, Aebersold R, Sonenberg N (2000) Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. EMBO J **19**: 434-444

Raught B, Gingras A-C (2007) Signaling to Translation Initiation. In Mathews, M.B., Sonenberg, N., and Hershey, J.W.B., editors *Translational Control in Biology and Medicine*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 369-400

Raught B, Peiretti F, Gingras AC, Livingstone M, Shahbazian D, Mayeur GL, Polakiewicz RD, Sonenberg N, Hershey JW (2004) Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J **23**: 1761-1769

Reed JC, Bidwai AP, Glover CVC (1994) Cloning and Disruption of Ckb2, the Gene Encoding the 32-Kda Regulatory Beta'-Subunit of Saccharomyces-Cerevisiae Casein Kinase-Ii. J Biol Chem **269**: 18192-18200

Reibarkh M, Yamamoto Y, Singh CR, del Rio F, Fahmy A, Lee B, Luna RE, Ii M, Wagner G, Asano K (2008) Eukaryotic initiation factor (eIF) 1 carries two distinct eIF5-binding faces important for multifactor assembly and AUG selection. J Biol Chem **283**: 1094-1103

Riera M, Pages M, Issinger OG, Guerra B (2003) Purification and characterization of recombinant protein kinase CK2 from Zea mays expressed in Escherichia coli. Protein Expr Purif **29**: 24-32

Riera M, Peracchia G, de NE, Arino J, Pages M (2001a) Maize protein kinase CK2: regulation and functionality of three beta regulatory subunits. Plant J **25**: 365-374

Riera M, Peracchia G, Pages M (2001b) Distinctive features of plant protein kinase CK2. Mol Cell Biochem **227**: 119-127

Roux PP, Blenis J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev **68**: 320-344

Salinas P, Bantignies B, Tapia J, Jordana X, Holuigue L (2001) Cloning and characterization of the cDNA coding for the catalytic alpha subunit of CK2 from tobacco. Mol Cell Biochem **227**: 129-135

Salinas P, Fuentes D, Vidal E, Jordana X, Echeverria M, Holuigue L (2006) An extensive survey of CK2 alpha and beta subunits in Arabidopsis: Multiple isoforms exhibit differential subcellular localization. Plant Cell Physiol **47**: 1295-1308

Sarno S, Ghisellini P, Pinna LA (2002) Unique activation mechanism of protein kinase CK2 - The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the holoenzyme. J Biol Chem **277**: 22509-22514

Scheper GC, Van Kollenburg B, Hu JZ, Luo YJ, Goss DJ, Proud CG (2002) Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J Biol Chem **277**: 3303-3309

Schmitt E, Blanquet S, Mechulam Y (2002) The large subunit of initiation factor alF2 is a close structural homologue of elongation factors. EMBO J **21**: 1821-1832

Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J, Hershey JW, Blenis J, Pende M, Sonenberg N (2006) The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO J **25**: 2781-2791

Shaikhin SM, Smailov SK, Lee AV, Kozhanov EV, Iskakov BK (1992) Interaction of wheat germ translation initiation factor 2 with GDP and GTP. Biochimie **74**: 447-454

Shen JJ, Pavone A, Mikulec C, Hensley SC, Traner A, Chang TK, Person MD, Fischer SM (2007) Protein expression profiles in the epidermis of cyclooxygenase-2 transgenic mice by 2-dimensional gel electrophoresis and mass spectrometry. Journal of Proteome Research **6**: 273-286

Singh CR, Yamamoto Y, Asano K (2004) Physical association of eukaryotic initiation factor (eIF) 5 carboxyl-terminal domain with the lysine-rich eIF2 beta segment strongly enhances its binding to eIF3. J Biol Chem **279**: 49644-49655

Singh LP, Aroor AR, Wahba AJ (1994) Phosphorylation of the guanine nucleotide exchange factor and eukaryotic initiation factor 2 by casein kinase II regulates guanine nucleotide binding and GDP/GTP exchange. Biochemistry **33**: 9152-9157

Sugano S, Andronis C, Green RM, Wang ZY, Tobin EM (1998) Protein kinase CK2 interacts with and phosphorylates the Arabidopsis circadian clock-associated 1 protein. Proc Natl Acad Sci USA **95**: 11020-11025

Sugano S, Andronis C, Ong MS, Green RM, Tobin EM (1999) The protein kinase CK2 is involved in regulation of Circadian rhythms in Arabidopsis. Proc Natl Acad Sci USA **96**: 12362-12366

Szebeni A, Hingorani K, Negi S, Olson MOJ (2003) Role of protein kinase CK2 phosphorylation in the molecular chaperone activity of nucleolar protein B23. J Biol Chem **278**: 9107-9115

Timmer RT, Benkowski LA, Schodin D, Lax SR, Metz AM, Ravel JM, Browning KS (1993) The 5' and 3' untranslated regions of satellite tobacco necrosis virus RNA affect translational efficiency and dependence on a 5' cap structure. J Biol Chem **268**: 9504-9510

Tokuda Y, Taki M, Sisido M (2006) Efficient incorporation of a nonnatural amino acid into a protein in an insect cell-free translation system. Nucleic Acids Symp Ser (Oxf) **132**: 277-278

Tsai SC, Seto E (2002) Regulation of histone deacetylase 2 by protein kinase CK2. J Biol Chem **277**: 31826-31833

Valásek L, Mathew AA, Shin BS, Nielsen KH, Szamecz B, Hinnebusch AG (2003) The yeast eIF3 subunits TIF32/a, NIP1/c, and eIF5 make critical connections with the 40S ribosome in vivo. Genes & Dev **17**: 786-799

Valasek L, Nielsen KH, Hinnebusch AG (2002) Direct eIF2-eIF3 contact in the multifactor complex is important for translation initiation in vivo. EMBO J **21**: 5886-5898

Valasek L, Nielsen KH, Zhang F, Fekete CA, Hinnebusch AG (2004) Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. Mol Cell Biol **24**: 9437-9455

Valero E, Chambaz EM, Cochet C (1997) Modulation of the protein kinase CK2 activity by a synthetic peptide corresponding to the N-terminus of its beta regulatory subunit. Biochem Biophys Res Commun **232**: 178-182

Van Drogen F, Peter M (2002) MAP kinase cascades: Scaffolding signal specificity. Curr Biol **12**: R53-R55

Vornlocher HP, Hanachi P, Ribeiro S, Hershey JWB (1999) A 110-kilodalton subunit of translation initiation factor eIF3 and an associated 135-kilodalton protein are encoded by the *Saccharomyces cerevisiae TIF32* and *TIF31* genes. J Biol Chem **274**: 16802-16812

Wang GX, Unger G, Ahmad KA, Slaton JW, Ahmed K (2005) Downregulation of CK2 induces apoptosis in cancer cells - A potential approach to cancer therapy. Mol Cell Biochem **274**: 77-84

Wang XM, Paulin FEM, Campbell LE, Gomez E, O'Brien K, Morrice N, Proud CG (2001) Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the e-subunit and their functions *in vivo*. EMBO J **20**: 4349-4359

Webb BLJ, Proud CG (1997) Eukaryotic initiation factor 2B (eIF2B). Int J Biochem Cell Biol **29**: 1127-1131

Wek RC, Jiang HY, Anthony TG (2006) Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans **34**: 7-11

Xu X, Toselli PA, Russell LD, Seldin DC (1999) Globozoospermia in mice lacking the casein kinase II alpha ' catalytic subunit. Nature Genet **23**: 118-121

Yahalom A, Kim TH, Winter E, Karniol B, Von Arnim AG, Chamovitz DA (2001) Arabidopsis eIF3e (INT-6) Associates with Both eIF3c and the COP9 Signalosome Subunit CSN7. J Biol Chem **276**: 334-340

Yamamoto Y, Singh CR, Marintchev A, Hall NS, Hannig EM, Wagner G, Asano K (2005) The eukaryotic initiation factor (eIF) 5 HEAT domain mediates multifactor assembly and scanning with distinct interfaces to eIF1, eIF2, eIF3, and eIF4G. Proc Natl Acad Sci U S A **102**: 16164-16169

Yan TF, Tao M (1982a) Purification and characterization of a wheat germ protein kinase. J Biol Chem **257**: 7037-7043

Yan TF, Tao M (1982b) Studies on an endogenous substrate of wheat germ protein kinase. J Biol Chem **257**: 7044-7049

Yatime L, Schmitt E, Blanquet S, Mechulam Y (2004) Functional molecular mapping of archaeal translation initiation factor 2. J Biol Chem **279**: 15984-15993

Zanchin NIT, McCarthy JEG (1995) Characterization of the In-Vivo Phosphorylation Sites of the Messenger-Rna-Center-Dot-Cap-Binding Complex Proteins Eukaryotic Initiation Factor-4e and P20 in Saccharomyces-Cerevisiae. J Biol Chem **270**: 26505-26510

Zhang CJ, Vilk G, Canton DA, Litchfield DW (2002) Phosphorylation regulates the stability of the regulatory CK2 beta subunit. Oncogene **21**: 3754-3764

Zhou C, Labbe H, Sridha S, Wang L, Tian L, Latoszek-Green M, Yang Z, Brown D, Miki B, Wu K (2004) Expression and function of HD2-type histone deacetylases in Arabidopsis development. Plant J **38**: 715-724

# Vita

Michael Don Dennis was born in Paducah, Kentucky on June 21, 1980, to Denny C. Dennis and Ginger L. Webster. He attended Centre College where he graduated *magna cum laude* with a bachelor degree in Biology in 2002. In 2003, he went on to pursue doctoral studies in biochemistry at the University of Texas, under the supervision of Professor Karen S. Browning.

Permanent Address: 526 Westbury Lane, Georgetown, TX 78633

This dissertation was typed by the author.